REGULATION OF INTRACELLULAR SIGNALLING LEADING TO GENE EXPRESSION IN LIPOPOLYSACCHARIDE-STIMULATED MURINE MACROPHAGES

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UNIFORMED SERVICES UNIVERSITY OF THE HEALTH SCIENCES
4301 JONES BRIDGE ROAD
BETHESDA, MARYLAND 20814-4799

GRADUATE EDUCATION
(301) 295-3913

APPROVAL SHEET

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Name of Candidate: Sheila Barber
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William C. Raum Committee Chairperson	9/20/95 Date
Stefanie N. Vogel Committee Member	9/27/95 Date
Susan D. Langevin Committee Member	9/20/95 Date
Carl W. Dufficl Committee Member	9/20/95 Date

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Sheila A. Barber

Department of Microbiology and Immunology

Uniformed Services University
of the Health Sciences

ABSTRACT

Title of Dissertation: Regulation of Intracellular Signaling Leading to Gene Expression in Lipopolysaccharide-Stimulated Murine Macrophages

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Due to the ubiquitous nature of LPS and its causative relationship to Gram negative sepsis, the study of LPS-induced cellular responses has been an area of active research for well over a decade. Earlier studies revealed that LPS stimulation results in the production of a cascade of proinflammatory mediators, such as cytokines. Recent studies have attempted to unravel the intricacies of the LPS signaling pathways that lead to cytokine production, in the hope of uncovering potentially vulnerable targets for pharmacologic intervention in septic shock. The studies presented herein were designed with such a purpose in mind. At the cellular level, manipulation of the LPS response in macrophages may occur at the plasma membrane, in the cytoplasm, or in the nucleus, and thus, we examined the regulation of LPS-induced gene expression at each of these levels. The studies of nuclear regulation focused on an evaluation of the role of Interferon Regulatory Factors (IRFs) in LPS-induced gene expression. The results suggest that while IRFs contribute to the regulation of LPS-inducible genes, the development of agents that may inhibit the function of these proteins may be of little use in combating sepsis, as LPS-induced immediate-early cytokine gene expression is essentially unaffected in macrophages derived from IRF "knock-out" mice.

Experiments aimed at examining regulation at the cytoplasmic level revealed the novel dependence of LPS-induced signaling on serine/threonine phosphatase activity. Furthermore, these studies identified a compound, calyculin A, that could mediate the inhibition of LPS signaling. This finding is particularly important for two reasons. First, if the observations extend to human macrophages, the development of a non-toxic form of calyculin A may prove to be efficacious for the prevention of septic shock. Secondly, calyculin A may now be used as an experimental tool to probe for the serine/threonine phosphatase critical for LPS-signaling. Our studies of LPS signal regulation at the plasma membrane revealed a remarkable coincidence: not only is the cellular response to endotoxin entirely dependent on the expression of a functional *Lps* gene product, but the ability of cells to activate an independently stimulated second messenger pathway, the ceramide-activated pathway, is also entirely dependent on the expression of the *Lps* gene product. This finding provides yet another direction from which to pursue the identity of the elusive *Lps* gene.

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by

Sheila A. Barber

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For me, the pupose of this section is to acknowledge the special people who also contributed to this work by influencing my development as an individual, family member, and friend. To these eight special people, listed below in the chronological order in which they influenced my life, I say, "Thank you"...

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Dad-- For imparting in me, the importance of honesty, integrity, and education

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In addition, an extra "Thank you" is extended to those below for the gift of personal freedom that we share, through the mutual acceptance and support of our individualities without judgement.

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DEDICATION

To A.P.S. and the "Tunes", past, present, and future

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ABBREVIATIONS

17-10 - LPS-inducible gene encoding a transription factor

-/- - designation indicating a targeted disruption of a gene on both chromosomes

bp - base pairs

B-rel - a member of the NF-κB/Rel family of DNA binding proteins

cDNA - complementary deoxyribonucleic acid

c-fos - proto-oncogene that encodes transcription factor Fos

CHX - cycloheximide

c-jun - proto-oncogene that encodes transcription factor Jun

c-myc - proto-oncogene that encodes transcription factor Myc

CO_2 - carbon dioxide

c-raf - proto-oncogene that encodes raf-1, a member of the "MAP kinase kinase kinase" family of ser/thr kinases

c-rel - proto-oncogene that encodes Rel, a member of the NF-κB/Rel family of DNA binding proteins

DTT - dithiothreotol

EDTA - disodium ethylene diamine tetraacetate · $2H_2O$

egr-1 - early growth response gene -1

EGTA - ethylene glycol bis-(β-aminoethyl ether) N,N,N'-tetraacetic acid

Elk-1 - member of the Ets family of transcription factors

Ets - E-twenty six

FcγR - Fc receptor for IgG on macrophages

FCS - fetal calf serum

h - hours

HEPES - N-2-hydroxy-ethyl piperazine-N'-2-ethanesulfonic acid

ICSBP - Interferon Consensus Sequence Binding Protein

IFN - Interferon

IFN-α/β/γ - Interferon-alpha/beta/gamma

IgG - Immunoglobulin G

IL - Interleukin

iNOS - inducible form of nitric oxide synthase

IP-10 - Interferon-γ-inducible protein 10

IRF - Interferon Regulatory Factor

JE - intercrine family member

KC - intercrine family member

kDa - kilodalton

LAL - *Limulus* amoebocyte lysis activity

LPS - lipopolysaccharide

Lps - lipopolysaccharide response gene

Lps^d - lipopolysaccharide response gene, defective allele

Lps^n - lipopolysaccharide response gene, normal allele

mM - milimolar

mAb - monoclonal antibody

MAP - mitogen-activated protein

min - minutes

mRNA - messenger ribonucleic acid

µg - microgram

µl - microliter

µM - micomolar

M-MLV - Moloney Murine Leukemia Virus

MW - molecular weight

NF-κB - nuclear factor kappa-B

ng - nanogram

nm - nanometer

nM - nanomolar

$NO^{\cdot}$ - nitric oxide

NO_2^- - nitrite

NP-40 - Nonidet P-40

p40 - inducible chain of Interleukin-12

PAGE - polyacrylamide gel electrophoresis

PKC - Protein kinase C

PMSF - phenylmethylsulfonyl fluoride

rIFN-γ - recombinant interferon gamma

RNA - ribonucleic acid

RT - reverse transcriptase

SAP-1 - member of the Ets family of transcription factors

ser - serine

SDS - sodium dodecyl sulfate

SEM - standard error of the mean

SSC - sodium chloride-sodium citrate buffer

SMase - Sphingomyelinase (*Staphylococcus aureus*)

TBE - tris-borate-disodium ethylene diamine tetraacetate · $2H_2O$ buffer

T_H1 - T-helper cell type 1

T_H2 - T-helper cell type 2

thr - threonine

TKI - tyrosine kinase inhibitor(s)

TNF-α - tumor necrosis factor alpha

TP - tyrosine phosphorylation

TPCK - $C_{17}H_{18}ClNO_3S$

tyr - tyrosine

v-raf - viral gene encoding the "active form" of Raf-1

INTRODUCTION

Sepsis, Macrophages, and Cytokines

Sepsis is a very serious and relevant health care issue world-wide. Three recent independent reports (Parrillo *et al.*, 1993; Bone *et al.*, 1993; Stone, 1994) have estimated that between 300,000 - 400,000 cases of sepsis occur annually in the United States alone, resulting in approximately 175,000 deaths. The "sepsis syndrome" is actually a severe systemic inflammatory response to infection that is mediated by potent soluble proteins such as cytokines (Sibbald *et al.*, 1995). When the infection is due to Gram negative organisms, the lipopolysaccharide (LPS) released from the surface of the bacteria, and in particular, the lipid A region of the LPS, initiates the massive inflammatory response (Glauser *et al.*, 1991). Twenty to fifty percent of the total number of sepsis cases result from Gram negative infection (Bone *et al.*, 1993; Natanson *et al.*, 1994). The most severe condition of sepsis, termed septic shock, is characterized by profound hypotension leading to organ perfusion, multiple organ failure, and frequently, death (Cohen *et al.*, 1991; Glauser *et al.*, 1991).

Several lines of evidence support the hypothesis that macrophages play a critical role in mediating sepsis. First, LPS-stimulated macrophages produce *in vitro* virtually all of the cytokines elicited *in vivo* upon LPS administration (Dinarello et al., 1986; Cohen *et al.*, 1991; Glauser *et al.*, 1991; Natanson *et al.*, 1994). Secondly, mice that are genetically hyporesponsive to LPS (i.e., they express the Lps^d allele, see below) can be rendered LPS-responsive upon adoptive transfer of macrophage progenitors (Michalek *et al.*, 1980) or mature macrophages (Freudenberg *et al.*, 1986) derived from fully

LPS-responsive (*Lps*^{n}) mice. In addition, mice with other profound defects in their lymphoid compartments [e.g., T cell-deficient (nu/nu) (Vogel *et al.*, 1979) mice, B cell-deficient (CBA/N xid) mice (Rosenstreich *et al.*, 1978), severe combined immunodeficient (SCID) mice (Falk *et al.*, 1995) and splenectomized mice (Madonna and Vogel, 1986)] are all normally sensitive to LPS administration. Finally, a recent examination of cytokine gene expression in LPS-stimulated, macrophage-depleted mice, revealed that macrophages are the major cellular source of IL-1β, IL-6, IL-10, IL-12 (the inducible chain, p40), and TNF-α mRNA produced in the liver (Salkowski *et al.*, 1995). Furthermore, these studies showed that macrophages were the primary producers of LPS-induced IL-1β, IL-6, and IL-12 mRNA in the spleen.

Cytokines are considered to be the direct mediators of the pathophysiology associated with septic shock for two main reasons. First, injection of purified recombinant cytokines can elicit LPS-like symptoms, and secondly, the lethal effects of LPS can be reversed by administration of certain cytokine antagonists (reviewed in Vogel, 1992). *In vivo*, cytokines are induced by LPS in a temporal, hierarchical fashion, that is often referred to as a "cytokine cascade" (reviewed in Vogel and Hogan, 1990). Historically, the earliest LPS-inducible products, arising one to six hours after LPS exposure (Griesman and Woodward, 1965; Sipe and Rosenstreich, 1981; and Sipe, 1990), were termed "acute phase reactants," and include Tumor Necrosis Factor (TNF; Carswell *et al.*, 1975; Helson *et al.*, 1975; Dinarello *et al.*, 1986; Beutler and Cerami, 1987), Interleukin (IL)-1 (reviewed in Kampschmidt *et al.*, 1973; Oppenheim *et al.*, 1991; reviewed in Dinarello, 1995), Interferon (IFN; Youngner and Stinebring, 1965; Youngner and Feingold, 1967; Ho *et al.*, 1967; Ho *et al.*, 1970; reviewed in Vogel, 1992; Cockfield, *et al.*, 1993), IL-6 (Van Snick

et al., 1986; Aarden *et al.*, 1987), IL-8 (Oppenheim *et al.*, 1991; Van Zee *et al.*, 1991, Porat *et al.*, 1992), and Colony Stimulating Factor (CSF; Pluznik, 1983; Williams *et al.*, 1983). TNF and IL-1 are "endogenous pyrogens" that induce fever via a prostaglandin-mediated effect on the hypothalamus (Atkins and Wood, 1955; Atkins, 1960; Snell and Atkins, 1968; Dinarello *et al.*, 1986). TNF and IL-1 also mediate LPS-induced hypoglycemia (Bauss *et al.*, 1987; Del Ray and Besedofsky, 1987; Vogel *et al.*, 1990) and the induction of "late phase reactants" (Sipe and Rosenstreich, 1981), that appear after 18 - 24 hours following LPS exposure. These include fibrinogen, C-reactive protein, and serum amyloid A (Sipe, 1990). TNF has also been implicated in LPS-induced hypotension (Floch *et al.*, 1989; Rabinovici *et al.*, 1990; Rabinovici *et al.*, 1991). Since N^G-methyl-L-arginine, an inhibitor of nitric oxide ($NO^{\bullet}$) production, has been shown to inhibit TNF-induced hypotension, $NO^{\bullet}$ is felt to be a more proximal mediator of endotoxemic hypotension (Kilbourn *et al.*, 1990). Strengthening this possibility was the finding that combinations of LPS and IFN-$\alpha/\beta/\gamma$ or IFN-γ and TNF/IL-1 synergize to induce $NO^{\bullet}$ (reviewed in Nathan, 1992); each of these cytokines is inducible by LPS *in vivo* (reviewed in Vogel, 1992). The recent availability of mice with targeted disruptions in the iNOS gene (i.e., the gene that encodes the $NO^{\bullet}$ synthase which, in turn, catalyzes the formation of $NO^{\bullet}$) confirmed a role for $NO^{\bullet}$ in LPS-induced hypotension, by demonstrating that $iNOS^{-/-}$ mice survived LPS challenge and suffered only a 15% decrease in arterial blood pressure, compared to the control group ($iNOS^{+/+}$) that died shortly after an LPS-induced 64% decrease in arterial blood pressure (MacMicking *et al.*, 1995). This study further demonstrated the existence of $NO^{\bullet}$-dependent and $NO^{\bullet}$-independent mechanisms of LPS-induced hypotension depending on the experimental inducers of endotoxemia.

It is clear from the above discussion, that cytokines are important mediators of LPS-stimulated effects. Hence, the development of reagents that interfere with the cytokine cascade have offered hope for intervention in sepsis. In this regard, the use of IL-1 receptor antagonist (Alexander *et al.*, 1991) or antibodies directed against TNF (Beutler *et al.*, 1985) and IFN-γ (Doherty *et al.*, 1992; Kohler *et al.*, 1993), have been found to protect mice from lethal doses of endotoxin. From the opposite perspective, other researchers have achieved success in preventing endotoxemic lethality in mice by administering certain "counteractive" cytokines (e.g., IL-10, IFN-α, G-CSF) that combat the LPS-induced cytokine cascade, especially by reducing the production of TNF (Görgen *et al.*, 1992; Tzung *et al.*, 1992; Gerard *et al.*, 1993).

Models of LPS-hyporesponsiveness

Sometime between 1960 and 1965, a spontaneous mutation occurred within the C3H/HeJ subline at Jackson Laboratories that resulted in a mouse strain that was refractory to LPS (reviewed in Vogel, 1992). The LPS-hyporesponsive C3H/HeJ mouse strain differs genetically from the closely related LPS-responsive C3H/OuJ strain at the *Lps* locus on Chromosome 4 (Watson *et al.*, 1978). To date, the *Lps* gene has not been cloned and it is not known if the *Lps* gene is a single gene or among a family of genes that maps to the same region on Chromosome 4. In any case, genetic studies support the hypothesis that responsiveness to LPS is controlled by a single, autosomal, co-dominantly inherited gene. As a result, macrophages (as well as other cell types) derived from C3H/HeJ mice, that express the Lps^d allele, do not respond to LPS, *in vivo* or *in vitro*, to produce cytokines (e.g., IFN, TNF, IL-1,

etc.) or other inflammatory mediators (e.g., IP-10, PGE_2, etc.) that are characteristic of normally LPS-responsive, C3H/OuJ (*Lps*^{n}) macrophages (reviewed in Vogel, 1992). In addition, *Lps*^{d} macrophages appear to be functionally less differentiated than *Lps*^{n} macrophages, as evidenced by their reduced FcγR (i.e., the Fc receptor for IgG on macrophages) capacity, that is reversible by the addition of exogenous IFNs (Fertsch *et al.*, 1984; Leu *et al.*, 1989). *Lps*^{d} macrophages, derived from conventionally-reared mice, are also permissive for viral replication, while *Lps*^{n} macrophages are resistant, a phenotype that is also reversible by prior treatment of *Lps*^{n} macrophages with antibodies specific for IFN-α/β (Vogel *et al.*, 1986; Vogel *et al.*, 1987). Based on these observations and the findings of Gessani *et al.* (1987), that supernatants from *Lps*^{n} macrophages, but not *Lps*^{d} macrophages, confer antiviral activity to aged macrophages, the hypothesis was developed that *Lps*^{n} macrophages maintain higher endogenous levels of IFN as a result of their ability to respond to the LPS present in normal Gram negative microbial flora, and that these LPS-inducible IFNs underlie the observed phenotypic differences in macrophage function. The principal species of IFN produced in response to LPS has been demonstrated serologically to be IFN-β, although IFN-α, and most recently, IFN-γ species have been detected at both the mRNA and protein levels (Havell *et al.*, 1983; Bellardelli *et al.*, 1987; Vogel, 1992; Fultz *et al.*, 1993).

A second model of endotoxin hyporesponsiveness can be found in normal mice that have been rendered transiently refractory to LPS by prior administration of a sublethal dose of LPS. This state of refractoriness is known as "endotoxin tolerance" (Favorite and Morgan, 1942; 1946; Greisman *et al.*, 1983). "Early endotoxin tolerance" induced by a single exposure to LPS

is cell-mediated (Greisman and Hornick, 1976; Williams *et al.*, 1983), and can be adoptively transferred with macrophages (Freudenberg *et al.*, 1987) to naive mice. Like the C3H/HeJ model, mice rendered endotoxin tolerant are highly refractory to LPS challenge and produce significantly diminished levels of LPS-induced cytokines (e.g., TNF, IL-1, IFN-β, IL-6, etc.; Madonna, *et al.*, 1985; Henricson *et al.*, 1990). Many years ago, it was also shown that macrophages derived from endotoxin tolerant mice were poor producers of "endogenous pyrogen" (now known to be IL-1 and TNF) and PGE_2 *in vitro* (Dinarello *et al.*, 1968; 1978; Reitschel *et al.*, 1980; Dinarello and Bernheim, 1981; Knudsen *et al.*, 1986). Within the past six years, an *in vitro* model of macrophage LPS-tolerance was developed by Virca *et al.* (1989). In this model, macrophages pre-exposed to LPS *in vitro* fail to respond to subsequent LPS "challenge" *in vitro* to produce TNF, thus mimicking macrophages rendered endotoxin tolerant *in vivo*. Despite much research, the fundamental mechanisms underlying endotoxin tolerance remain elusive.

SIGNALING MECHANISMS IN LPS-STIMULATED MACROPHAGES

The interaction of LPS with the macrophage involves coordinate transfer of extracellular signals to intracellular mediators that ultimately orchestrate the induction of genes that encode a wide variety of proteins including cytokines (IFN α/β/γ, TNF-α, IL-1β, IL-6, IL-10, IL-12), cytokine receptors (type 2 TNF receptor (TNFR-2), pro-inflammatory proteins [IFN-γ-inducible protein 10 (IP-10)], enzymes (e.g., iNOS), and transcription factors (c-*jun*, c-*fos*, c-*myc*; reviewed by Hamilton *et al.*, 1993). Induction and regulation of the signaling pathways elicited in LPS-stimulated macrophages is the subject of the research described herein. There are three major sites in macrophages at which LPS-induced signaling leading to gene expression may

be regulated: at the nucleus, the cytoplasm, and the plasma membrane. The studies presented in this dissertation were designed to examine potential regulatory mechanisms at each of these levels.

Nuclear Regulation of LPS-inducible Gene Expression

Nuclear regulation of gene expression typically involves sequence-specific DNA binding proteins that regulate transcription either by promoting or repressing the function of cellular transcription machinery. In many cases, LPS stimulation of macrophages results in an increase in the transcriptional activity of specific genes such as IP-10, JE, and KC ("competence genes"), TNF-α, IL-1β, D3, and D8 (the latter two are yet undefined LPS-inducible genes; reviewed in Hamilton *et al.*, 1993). However, other levels of gene regulation have been described. For example, LPS-inducible IFN-β mRNA accumulates by post-transcripitonal mechanisms (Gessani *et al.*, 1991). In addition, although LPS induces TNF-α mRNA via increased transcriptional activity (Beutler *et al.*, 1986), LPS also induces a time-dependent decrease in TNF-α mRNA stability, suggesting that both transcriptional and post-transcriptional mechanisms regulate LPS-inducible TNF-α (Han *et al.*, 1991). Moreover, LPS has been shown to induce translational derepression of translationally inactive forms of TNF-α mRNA present in unstimulated cells, suggesting yet another level at which LPS regulates TNF-α expression (Han *et al.*, 1990).

LPS-Inducible Transcription Factors

Previous studies of LPS stimulation have demonstrated that some of the immediate-early genes encode transcription factors c-*fos*, c-*jun*, and c-*myc*, egr-1, and 17-10, perhaps implicating these proteins in the regulation of other LPS-inducible genes (reviewed in Adams, 1992; Coleman *et al.*, 1992; Drysdale

et al., 1995). Other approaches, utilizing electrophoretic mobility shift assays, have led to a more direct identification of LPS-inducible transcription factors.

The proteins that constitute the transcription factor nuclear factor kappa-B (NF-κB) belong to the *rel* family (based on sequence homology) and include c-rel, B-rel, I-rel, p49, p50, and p65 (reviewed in Baeuerle *et al.*, 1994). The various heterodimeric complexes formed by these *rel* proteins result in distinct DNA binding (the consensus "κB site" is 5′-GGGANNYYCC-3′) and transactivation functions. Cytoplasmic activation of NF-κB results after the phosphorylation-dependent release and degradation of the inhibitory subunit IκB-α (Henkel *et al.*, 1993). NF-κB then translocates to the nucleus where it has been shown to bind specific sequences of DNA and to interact with other proteins such as the TATA-binding protein of the transcription factor IID complex, leading to enhanced transcriptional activity (Kerr *et al.*, 1993). LPS stimulation has been shown to induce the DNA binding of three complexes that contain p50, p50/p65, and c-*rel*, respectively, in a murine macrophage-like cell line (Ohmori *et al.*, 1994), whereas p50/p65 heterodimers predominate in LPS-stimulated murine peritoneal macrophages (Narumi *et al.*, 1992).

AP-1 is a heterodimeric transcription factor that is composed of the products of the proto-oncogenes c-*jun* and c-*fos*. A potential role for AP-1 in the LPS-induction of cytokine genes has been suggested (Reimann *et al.*, 1994) based on the integration of three independent observations: (1) AP-1 binding sites are extremely important for transcriptional activation of human TNF-α and IL-1β genes (Bensi *et al.*, 1990; Rhoades *et al.*, 1992); (2) A BAC macrophage cell line transformed with *v-raf*, an active form of *c-raf* (an LPS-inducible serine/threonine kinase that will be described below), contain

constitutive AP-1 binding activity (Büscher *et al.*, 1993); and, (3) *v-raf*-transformed BAC macrophages express constitutive TNF-α and IL-1β mRNA (Reimann *et al.*, 1994). A recent study identified LPS-inducible DNA binding activity of transcription factors *jun* and CREB to a lipopolysaccharide response element (LRE), that will be described below (Shin *et al.*, 1994).

The E-twenty six (Ets) family of transcription factors are diverse DNA binding proteins that recognize a purine-rich motif centered around a conserved GGA trinucleotide (reviewed in Macleod *et al.*, 1992). Ets proteins interact with other DNA binding proteins (Pongubala *et al.*, 1993) and exhibit distinct binding specificities to Ets motifs, based on sequences present in the flanking regions (Wang *et al.*, 1992). LPS stimulation of murine macrophages leads to the DNA binding activity of Ets proteins PU.1, Elk-1 and SAP-1 (Shackelford *et al.*, 1995; Reimann *et al.*, 1994). Elk-1 and SAP-1 have been shown to interact with the serum response elements (SRE) in the promoters of immediate-early genes, such as *fos*, by forming ternary complexes with dimeric serum response factor (SRF; Macleod *et al.*, 1992; Shore *et al.*, 1994). PU.1 is expressed only in B cell and macrophage lineages and does not form ternary complexes with serum response factor (Macleod *et al.*, 1992).

LPS-Responsive Promoter Elements

Transcription factors recognize specific DNA sequences in the promoter regions of genes that are collectively referred to as *cis*-acting elements. Four *cis*-acting elements have been identified in the promoter region of the murine TNF-α gene that appear to mediate its LPS-induciblity: two κB-like sites and two CK-1 (*c*yto*k*ine-1) sites (Drouet *et al.*, 1991; Shakhov *et al.*, 1990; Collart *et al.*, 1990). The c-*rel* proteins that recognize the κB sites

have been described above. Transcription factors NF-kB and NF-GMa (nuclear factor-GMa) can bind CK-1 motifs (Drouet *et al.*, 1991; Shannon *et al.*, 1990; Kuczek *et al.*, 1991). Three *cis*-acting elements have been shown to mediate LPS-inducible IP-10 gene expression: two κB sites and one full-length, highly conserved, Interferon Stimulated Response Element (ISRE; Hamilton *et al.*, 1993). LPS has been shown to induce binding to the ISRE derived from the IP-10 promoter in murine peritoneal macrophages (Tebo *et al.*, 1992). Several *cis*-acting elements exist in the region of murine iNOS gene promoter that mediate LPS-inducibility. These include six imperfect (7/8) γ-interferon response elements (IRE), an X box, a TNF response element, an imperfect (7/10) GAS (IFN-γ-activated site), two NF-IL6, an Oct, and a perfect NF-κB site (Lowenstein *et al.*, 1993; Xie *et al.*, 1993). A novel *cis*-acting element in the promoters of MuRANTES and crg-2 genes, termed "lipopolysaccharide response element," was identified and shown to mediate the LPS-inducibility of these genes (Shin *et al.*, 1994). Three cis-acting elements upstream of the CSF gene, that contain the consensus sequence for NF-IL6 sites (Akira *et al.*, 1990), were found to bind an LPS-inducible DNA binding protein in murine macrophages (Nishizawa and Nagata, 1990).

Cytoplasmic Regulation of LPS-inducible Gene Expression

Cells have evolved a multitude of second messenger pathways to link specific extracellular signals to the appropriate nuclear responses. LPS stimulation of macrophages elicits a particular subset of these pathways, which are coordinately regulated to provide the specificity of the whole cell response. An understanding of how each pathway is regulated and how each pathway contributes to the LPS response in macrophages, may also provide

intervention strategies for the treatment of septic shock. Thus, cellular regulation of LPS-induced signaling continues to be an area of active research.

LPS-Inducible Phospholipid Metabolism

Phospholipid metabolism results in the activation of at least three second messengers. Phosphatidylinositol 4,5-bisphosphate (PtdIns(4,5)P_2 or "PIP_2") is an inositol lipid located in the inner leaflet of the plasma membrane, that is formed from inositol by the action of two phosphatidylinositol kinases (Berridge *et al.*, 1984). Cellular homeostasis involves a balance between these kinases and the phosphomonoesterases that remove the phosphate groups, forming again, inositol. Cell surface receptor ligations often induce a conformational change in the membrane that makes PtdIns(4,5)P_2 more accessible to phosphodiesterase (phosphoinositide-specific phospholipase C (PLC)), which cleaves PtdIns(4,5)P_2 into phosphatidylinosiol(1,4,5)P_3 (IP_3) and sn-1,2-diacylglycerol (DAG). PLC may also be activated by G protein-linked receptors (Berridge *et al.*, 1984). IP_3 has been shown to release Ca^{2+} from intracellular stores, thus increasing intracellular Ca^{2+} levels. Increased levels of intracellular Ca^{2+}, in turn, lead to the activation of Ca^{2+}-dependent enzymes (reviewed in Clapham, 1995). As the metabolism continues, IP_3 is converted to PtdIns(1,3,4,5)P_4 which is then converted to PtdIns(1,3,4,)P_3 and ultimately back to inositol (Adams et al., 1992). Second messenger PtdIns(1,3,4,5)P_4 mediates the entry of extracellular Ca^{2+}, again resulting in increased intracellular Ca^{2+} levels. DAG is a natural activator of Protein Kinase C (PKC; Nishizuka, 1992), whose involvement in LPS signaling will be discussed below. DAG can also be formed from phosphatidycholine (PC) by the action of PC-specific PLC, phospholipase D (PLD), and phospholipase A_2, and from ceramide, phosphatidylserine,

phosphatidylethanolamine and triacylglycerol (reviewed in Nishizuka, 1992; Ho *et al.*, 1994). Many signal transduction pathways activate PtdIns 3-kinase, which converts PtdIns(4,5)P_2 to PtdIns(3,4,5)P_3, thus suggesting that PtdIns(3,4,5)P_3 may act as a second messenger as well (Divecha *et al.*, 1995). LPS stimulation of macrophages has been shown to activate phospholipid metabolism, which results in the production of IP_3 and DAG (Prpic *et al.*, 1987). LPS also induces the metabolism of PC to DAG, which is thought to account for the majority of DAG produced during LPS signaling (Grove *et al.*, 1990; Adams *et al.*, 1992).

Ion Mobilization in LPS-Stimulated Macrophages

The concentration of intracellular ions is maintained in balance by membrane-associated ion-specific channels and pumps (Schroeder and Hedrich, 1989). Alterations in intracellular ion concentrations have been linked to lymphocyte and neutrophil activation (Weisman *et al.*, 1987; Cheung *et al.*, 1988; Tocci *et al.*, 1989; reviewed in Lewis, 1995). LPS stimulation of macrophages does not alter Na^+/K^+ flux; however, experimentally-induced alterations of Na^+/K^+ flux during LPS stimulation potentiates the expression of TNF-α, IP-10 and KC genes (Ohmori, *et al.*, 1991). As described above, phospholipid metabolism results in the production of second messengers that increase intracellular Ca^{2+} levels. Evidence that LPS induces an increase in intracellular Ca^{2+} levels has been controversial (Prpic *et al.*, 1987); however, experimentally-induced increases in intracellular Ca^{2+} levels do not result in cytokine gene expression, and therefore, high levels of intracellular Ca^{2+} cannot mimic LPS signaling (Ohmori *et al.*, 1992). Calmodulin is a Ca^{2+}-binding protein subunit for several cellular enzymes (including PKC) that has been shown to increase enzymatic substrate affinity

(Carafoli, 1987). Two studies have shown that Ca^{2+}/calmodulin antagonists suppress LPS-induced IL-1β, but increase LPS-induced IP-10 gene expression (Kovacs *et al.*, 1989; Ohmori *et al.*, 1992). Thus, Ca^{2+} acting through calmodulin, may be a necessary, but insufficient, component of the LPS signaling pathway.

Effect of Cyclic Nucleotides on LPS-Inducible Gene Expression

The cyclic nucleotides cAMP and cGMP are two second messengers that are generated from ATP, by adenylate cyclase, and GTP, by guanylate cyclase, respectively. cAMP and cGMP activate cAMP- or cGMP-dependent protein kinases, respectively. In addition, cGMP has been shown to regulate cation-selective channels, leading to altered monovalent ion concentrations (Zuker, 1995). Elevation of intracellular cAMP has suppressive effects on macrophage activities such as antigen presentation and phagocytosis (Tannenbaum *et al.*, 1989). In addition, LPS has been shown to inhibit adenylate cyclase in membranes prepared from the macrophage cell line $P388D_1$, thus maintaining low intracellular cAMP levels (Jakway *et al.*, 1986). In order to assess the involvement of cAMP in LPS signaling, several investigators have examined the effects of various agents that increase intracellular concentration of cAMP on LPS-inducible gene expression. The results indicated that elevated intracellular levels of cAMP have no effect on LPS-inducible levels of KC and IL-1α mRNA, suppress levels of TNF-α and JE mRNA, and enhance levels of IL-1β mRNA (Tannenbaum *et al.*, 1989; Nakano *et al.*, 1990; Ohmori *et al.*, 1990). In addition, elevated levels of cGMP had no effect on IL-1 α production (Nakano *et al.*, 1990). Thus, cyclic nucleotides appear not to mediate LPS signaling, but increased levels of cyclic nucleotides may regulate certain LPS-induced pathways.

Involvement of G proteins in LPS Signaling

G proteins (guanine nucleotide-binding proteins) are a family of receptor-associated signal transduction molecules (reviewed in Simon, *et al.*, 1991). The heterotrimeric G proteins consist of α, β, and γ subunits. Upon receptor ligation, the GDP-bound α subunit releases GDP in favor of GTP, dissociates from the β and γ subunits, and activates (Gsα) or inhibits (Giα) adenylate cyclase activity (Gilman, 1984). The α-subunits of Gi contain ADP-ribosylation sites for pertussis toxin, which effectively inhibits the activity of the Gi protein. Two Gi proteins, Gi_2 and Gi_3, are expressed in macrophages (Daniel-Issakani *et al.*, 1989). The initial suggestion that LPS may activate Gi, arose from two independent observations: (1) LPS-induced inhibition of adenylate cyclase activity could be blocked by pre-treatment with pertussis toxin; and (2) pre-treatment of adenylate cyclase-containing membranes prepared from the murine macrophage cell line, $P338D_1$, with LPS, abolished pertussis toxin-mediated ADP-ribosylation of Gi (Jakway *et al.*, 1986). Subsequently, it was shown that the LPS response in the human macrophage cell line, U937, was linked to Gi_2 (Daniel-Issakani *et al.*, 1989). Both studies demonstrated that pertussis toxin inhibited LPS-induced IL-1 production. More recent experiments revealed that pertussis toxin also inhibits LPS-inducible $NO^{\bullet}$ production, but enhanced LPS-induced TNF-α secretion (Zhang *et al.*, 1993). Taken collectively, LPS activation of G proteins appears to regulate certain LPS-initiated signaling pathways differentially.

Involvement of PKC in LPS Signaling

PKC is a Ca^{2+}-sensitive, phospholipid-dependent serine (ser)/threonine (thr) kinase (reviewed in Nishizuka, 1992). LPS may activate

PKC by three different mechanisms: (1) via DAG: LPS-induced phospholipid metabolism (see above) produces DAG, which is a natural activator of PKC, and has been shown to induce translocation of cytosolic PKC to the plasma membrane (see DAG above). In the presence of cofactor phosphatidylserine (PS), PKC has been shown to phosphorylate a number of substrates including transcription factors (*fos* and *jun*) and actin filament crosslinking protein, MARCKS (myristoylated, alanine-rich C kinase substrate; Bakouche *et al.*, 1992; Seykora *et al.*, 1991; Hartwig *et al.*, 1992); (2) directly: Lipid A was shown to activate directly crude PKC isolated from RAW 264.7 cells, and the activation was enhanced in the presence of DAG (Wightman *et al.*, 1984); and (3) via arachidonic acid: Arachidonic acid and other long chain fatty acids have been shown to stimulate PKC activity, and LPS has been shown to stimulate macrophages to metabolize arachidonic acid into potent inflammatory mediators called eicosanoids (Weiel *et al.*, 1986; McPhail *et al.*, 1984; Kurland *et al.*, 1978). The use of H7, a PKC inhibitor, was shown to inhibit LPS induced TNF-α (mRNA and protein) and IL-1β (protein) in murine macrophages and human monocytes (Kovacs *et al.*, 1988; Kovacs *et al.*, 1989; Shapira *et al.*, 1994). However, the interpretations of experiments using H7 (or staurosporine) are confounded by the lack of specificty of these inhibitors; both bind to the catalytic sites of PKA and PKG (protein kinases A and G), tyrosine kinases, and phosphorylase a kinase (Bakouche *et al.*, 1992). Using a different approach, another study assessed the role of PKC by using H7 in monocytes stimulated with either free LPS or liposome-incorporated LPS (Bakouche *et al.*, 1992). The results indicated that free LPS initiates translocation of cytosolic PKC to the plasma membrane, IL-1 production (i.e., cell-associated), and IL-1 secretion (i.e., in the supernatants). Liposome-incorporated LPS enters by endocytosis and induces IL-1 production, but not

translocation of PKC or IL-1 secretion. Free LPS, in the presence of H7, induces IL-1 production, but not translocation of PKC or IL-1 secretion. Thus, IL-1 production and secretion are regulated independently and only IL-1 secretion involves PKC. Collectively, the above studies strongly suggest the involvement of PKC in LPS signaling.

LPS-Inducible Phosphorylation

Enzyme-mediated phosphorylation/dephosphorylation of cellular proteins is a major regulatory mechanism by which extracellular signals can control intracellular functions. The original observation that demonstrated the LPS-induced phosphorylation of four proteins, p67, p37, p33 and p28, was made in 1986 (Weiel *et al.*, 1986). In this study, PMA (phorbol myristate acetate), a PKC activator, also induced the phosphorylation of these proteins, perhaps implicating PKC in the phosphorylation. By 1991, p65, a protein phosphorylated on a serine residue in response to LPS stimulation had been purified and characterized, and by 1995, the complete sequence and structure had been determined (Shinomya *et al.*, 1991; Shinomya *et al.*, 1995). "p65" is the murine homologue of L-plastin, a transformation-induced polypeptide in neoplastic human fibroblasts, and sequence analysis revealed consensus sites for cAMP-dependent PKA and PKC, but not for the MAP (mitogen-activated protein) kinases that will be described below.

LPS-Induced Tyrosine Phosphorylation of MAP Kinases

In addition to ser/thr residues, certain tyrosine (tyr) residues may also be phosphorylated in response to LPS. The first observation that LPS stimulation results in tyrosine phosphorylation (TP) was made in 1991 (Weinstein *et al.*, 1991). LPS-stimulation of RAW 264.7 cells, and *Lps*^{n}, but

not *Lps*^{d} macrophages, resulted in the TP of three proteins in the 40 - 45 kDa range. TP was maximal at 15 min after LPS stimulation and declined to basal levels by 30 - 60 min. The three proteins phosphorylated on tyrosine in response to LPS were subsequently identified as MAP kinases *erk* (extracellular signal-regulated kinase)-1, *erk*-2, and p38 (Weinstein *et al.*, 1992; Han *et al.*, 1994). The MAP kinases are a family of ser/thr kinases that become activated following phosphorylation on both thr and tyr residues (reviewed in Cobb and Goldsmith, 1995). Three subgroups of MAP kinases have been identified, the *erks*, p38, and the *jun* kinases (JNK), and these are distinguishable by the sequence motifs surrounding their dual phosphorylation sites (Dérijard *et al.*, 1995). Substates for MAP kinases include transcription factors *myc*, *jun*, ATF2, and Ets proteins (Seth *et al.*, 1991; Pulverer *et al.*, 1991; Gille *et al.*, 1992; Janknecht *et al.*, 1993; Gupta *et al.*, 1995), enzymes such as PLA_2 (Lin *et al.*, 1993), and other kinases such as ribosomal S6 kinase (*rsk*; Chen *et al.*, 1992). Researchers have recently been able to link MAP kinase activity to cellular functions. Lee *et al.* (1994) identified two new human MAP kinases, CSBP1, and CSBP2, and demonstrated that inhibition of the activation of these kinases resulted in translational inhibition of cytokine production. Furthermore, sequence analysis revealed that the murine homologue of CSBP2 is "p38".

The MAP kinases, themselves, are substrates for "MAP kinases kinases", which are "dual specificity" kinases (i.e., they can phosphorylate MAP kinases on both thr and tyr residues), that are, themselves, activated by phosphorylation on serine residues (Alessi *et al.*, 1994). The MAP kinase kinase family is currently comprised of four members, MEK1, MEK2, MKK3, MKK4 (Cobb and Goldsmith, 1995), and constitutively active MAP kinase

kinases have been shown to transform mammalian cells (Mansour *et al.*, 1994). The specificity of responses to different activators that stimulate particular MAP kinase pathways is achieved by hierarchical regulation. For example, activation of MEK1 or MEK2 results in preferential phosphorylation of the *erks*, and not p38 or JNK (Cobb and Goldsmith, 1995). Activation of MKK3 results in preferential activation of only p38, and activation of MKK4 results in preferential phosphorylation of JNK and p38, but not the *erks* (Dérijard *et al.*, 1995).

One approach that has been taken to assess the role of TP in LPS signaling is the use of tyrosine kinase inhibitors (TKI). For example, the tyrosine kinase inhibitor, radicol, was shown to suppress the expression of LPS-inducible cyclooxygenase enzyme that catalyzes the conversion of arachidonic acid to prostaglandins, in macrophages (Chanmugan *et al.*, 1995). Three other TKI, genestein, herbimycin A, and tyrphostin, were shown to inhibit LPS-inducible $NO^{\bullet}$ synthase activity (Dong *et al.*, 1993). Other studies have found that tyrosine phosphorylation is required for induction of macrophage tumoricidal activity (Dong *et al.*, 1993). Several groups have established that tyrosine kinase inhibitors block LPS-induced TNF-α production (Shapira *et al.*, 1994; Beaty *et al.*, 1994) simultaneously with the inhibition of MAP kinase TP (Dong *et al.*, 1993; Novogrodsky *et al.*, 1994), which led to the hypothesis that TP of MAP kinases is required for LPS-inducible TNF-α production in macrophages. In addition, one of these studies (Novogrodsky *et al.*, 1994), correlated inhibition of TNF-α and TP of MAP kinases with protection of mice from LPS-induced lethality.

LPS Induction of the Ras/Raf-1/MEK-1/MAP Pathway

Ras is the product of oncogene c-*ras*, and is a member of the GTPase superfamily (reviewed in Boguski and McCormick, 1993; Cahnt and Stowers, 1995). As such, Ras shares structural similarity with the Gsα subunits described above. Inactive Ras is bound to GDP, and upon activation, Ras exchanges GDP for GTP. GTP-bound Ras is an active ser/thr kinase, that has been shown to bind and localize members of the "MAP kinase kinase kinase" family, such as raf-1 and MEKK1 to the plasma membrane (Moodie, *et al.*, 1993; Herrmann *et al.*, 1994; Russell *et al.*, 1995). It is unclear how the MAP kinase kinase kinases are activated (Whitehurst *et al.*, 1995); however, two signals (one Ras-dependent and one Ras-independent) may be required (Fu *et al.*, 1994). Activated MEKK1 is the preferential activator of MAP kinase kinase MKK4 (described above), and activated raf-1 preferentially activates MAP kinase kinases MEK1 and MEK2 by phosphorylation on serine residues (described above; Alessi *et al.*, 1994; reviewed in Cobb and Goldsmith, 1995). Thus, another level of hierarchical regulation controls signal specificity through the MAP kinase pathway. Regulation of Ras activity involves the guanine nucleotide exchange factors, that facilitate the association of GDP-Ras with GTP, and GTPase-activating proteins (GAPs) that increase the rate of GTP hydrolysis (Boguski and McCormick, 1993). Constitutively active Ras transforms cells and, interestingly, overexpression of mutated Ets proteins (described above), that are able to bind DNA but not activate transcription, can revert Ras-transformed cells (Wasylyk *et al.*, 1994). Recently, Ras was shown to mediate the activation of PLD (described above) by v-*src* (Jiang *et al.*, 1995).

Two independent studies have implicated the raf-1/MEK/MAP kinase pathway in LPS signaling in macrophages (Geppert *et al.*, 1994; Reimann *et al.*,

1994). However, the involvement of Ras in LPS signaling is controversial. Geppert *et al.* (1994) showed that the use of a dominant inhibitor of Ras inhibited LPS-induced activation of the TNF-α promoter. In contrast, another study demonstrated that CSF-1, but not LPS, could stimulate Ras activity in macrophages (Büscher *et al.*, 1995). Ras-independent activation of raf-1 has been described very recently (Hawes, *et al.*, 1995; Hou *et al.*, 1995), and interestingly, high intracellular levels of cAMP (described above) have been shown to inhibit activation of the Raf-1/MEK/MAP pathway, but not activation of Ras, in response to EGF (epidermal growth factor; Cook and McCormick, 1993).

Involvement of Src-Related Kinases in LPS Signaling

Src-related kinases are membrane-associated phosphoproteins with tyrosine kinase activity (reviewed in Eiseman and Bolen, 1990). Nine such kinases have been described, which are differentially expressed in various cell types, but share a common structure that includes an N-terminal SH2 and SH3 domain. When the kinase is in an inactive state, the SH2 domain is thought to bind to a phosphotyrosine residue at the carboxy terminus, and the SH3 domain may also bind to the carboxy terminus, thereby maintaining conformational occlusion of the catalytic domain (reviewed in Cooper and Howell, 1993). Thus, *src*-related kinase activity may be regulated by tyrosine kinases and tyrosine phosphatases. Upon activation, the inactive kinase undergoes a conformational change, exposing the catalytic domain, after which phosphorylation or autophosphorylation may occur. The accessibility of a phosphotyrosine residue allows association of *src*-related kinases with other SH2 domains, such as that of adaptor protein Grb2 (described above; Egan *et al.*, 1993). Grb2 also contains two SH3 domains through which it

interacts with guanine nuclear exchange factor (described above) Sos, which then promotes the association of Ras with GTP. In this way, membrane signals involving phosphotyrosine residues may ultimately result in the activation of Ras. Such a mechanism has been described for EGF receptor signaling (Buday and Downward, 1993).

LPS signaling induces the transient activation of src-related kinases *lyn*, *hck*, and *fgr* (Stefanova *et al.*, 1993). In addition, it has been shown that *lyn* activation occurs in Lps^n, but not Lps^d macrophages, and that the LPS-induced autophosphorylation of *lyn* can be blunted by tyr phosphatase antagonists (Henricson *et al.*, 1995). Overexpression of *hck* has been found to augment LPS-induced TNF production whereas inhibition of *hck* interfered with LPS-inducible TNF production (English *et al.*, 1993). LPS has also been shown to induce the expression of the *lyn*, *hck*, and *fgr* genes in bone marrow-derived macrophages (Yi and Willman, 1989; Boulet *et al.*, 1991).

Regulation of LPS-Inducible Gene Expression at the Plasma Membrane

The eukaryotic plasma membrane mediates communication between the interior of the cell and the extracellular milieu. As a result of its biochemical properties it is likely that in order to initiate the signaling pathways described above, LPS interacts with a structure(s) associated with the plasma membrane. In addition, a number of lines of experimental evidence are consistent with the notion of multiple specific membrane-associated LPS-binding proteins or "LPS receptors" (described below, reviewed in Morrison, 1989; Ulevitch, 1993). Two major types of membrane proteins have been described: integral membrane proteins, that have one or more segments embedded in the membrane (Blobel, 1980), and peripheral membrane

proteins that associate with the membrane as the result of various lipid modifications such as myristoylation (e.g., *lyn*), palmitoylation (e.g., Ras), prenylation (e.g., trimeric G proteins), and glycosylphosphatidylinositol (GPI) linkage (CD14; reviewed in Casey, 1995). As described below, LPS has been suggested to interact with both major classes of membrane proteins. To date, the precise sequence of events that initiates LPS signaling across the plasma membrane remains elusive; however, significant progress has been made within the last five years toward elucidation of the molecular interactions involved in this process.

Lipopolysaccharide-Binding Proteins

About seventeen years ago, it was observed that high density lipoproteins (HDL) present in normal plasma, could alter certain biophysical properties of LPS (e.g., buoyant density), thus identifying HDL as a major LPS binding protein (Ulevitch and Johnston, 1978). A comparison of LPS binding to HDL in normal or acute phase serum led to the discovery of an LPS-binding protein present in acute phase serum, that became referred to as "LBP" (Tobias and Ulevitch, 1983; Tobias *et al.*, 1986; Schumann *et al.*, 1990). A role for LBP in LPS signaling was provided by studies that demonstrated that depletion of LBP from plasma resulted in decreased levels of LPS-induced TNF in whole rabbit blood (Schumann *et al.*, 1990). In addition, LBP was found to lower the minimal concentration of LPS required to stimulate rabbit macrophages and human monocytes and to enhance the rate of cytokine production (Heumann *et al.*, 1992; Mathison *et al.*, 1992; reviewed in Ulevitch and Tobias, 1995). Confirming the importance of LBP in LPS induced signaling was the finding that administration of antibodies specific for LBP was protective in mice when given simultaneously with lethal doses

of LPS (Gallay, *et al.*, 1993). Another LPS binding protein identified in normal plasma has been referred to as "septin" (Wright *et al.*, 1992), and both LBP and septin were shown to mediate LPS recognition by CD14 (Schumann *et al.*, 1990; Wright *et al.*, 1992), a protein that will be discussed below. LBP was found to accelerate the binding of LPS to sCD14 (Hailman *et al.*, 1994), and to facilitate the transfer of LPS to HDL (Wurfel *et al.*, 1994). HDLs function to neutralize LPS, and experimentally-induced elevation of HDL has been shown to protect animals from LPS-induced lethality (reviewed in Freudenberg and Galanos, 1992). Thus, it has been proposed that LBP both potentiates LPS signaling by facilitating the interaction of LPS with CD14, and aids in the neutralization of LPS by transferring LPS to HDL.

Macrophage-Associated LPS "Receptors"

A number of studies have demonstrated the existence of multiple, distinct LPS binding sites on human and murine monocyte/macrophages (Couturier *et al.*, 1991; Girard *et al.*, 1993). Of these, only four have been well characterized, and include the CD11/CD18 complexes, "the scavenger receptors", CD14, and a 73 kDa protein.

The CD11/CD18 leukocyte adhesion proteins are β2 integrins that share a common β subunit (CD18), but have distinct α subunits (CD11a, b, and c), and although CD11/CD18 complexes bind to cellular ligands, (ICAM-1, -2, and -3), they have also been shown to bind LPS present on the surface of bacteria and LPS-coated erythrocytes (Wright and Jong, 1986). That CD11/CD18 complexes are not essential mediators of LPS signaling was demonstrated using monocytes and macrophages deficient in CD18. In these studies, CD18-

deficient cells were shown to produce normal amounts of TNF-α and IL-1β in response to LPS (Wright *et al.*, 1990).

Receptors for acetylated low density lipoproteins (LDL) have been identified on the surface of macrophages, and these "scavenger receptors" have also been shown to bind lipid A (Hampton *et al.*, 1991). However, addition of acetylated LDL (i.e., the ligand for the scavenger receptors) inhibited the binding of lipid IV_A (the biological precursor of lipid A) to scavenger receptors and the subsequent internalization and metabolism of lipid IV_A, but not the lipid IV_A-induced stimulation of RAW 264.7 cells (Hampton *et al.*, 1991). Thus, it is currently accepted that scavenger receptors function in the clearance and detoxification of LPS (reviewed in Raetz *et al.*, 1991).

CD14

The best characterized LPS receptor is CD14. CD14 is a GPI-linked membrane protein (mCD14; Haziot *et al.*, 1988) expressed on the surface of monocytes, macrophages, and polymorphonuclear leukocytes (Goyert *et al.*, 1988). In addition, soluble forms of CD14 (sCD14) have been identified in serum and urine, although the mechanisms that account for this have not been well defined (Maliszewski *et al.*, 1985; reviewed in Ulevitch and Tobias, 1995). The first indication that CD14 may function as an "LPS receptor" arose from the observation that LBP/LPS complexes recognize mCD14 (Schumann *et al.*, 1990); however, it was noted that high concentrations of LPS could stimulate cells in the absence of detectable binding to CD14 (Wright *et al.*, 1990). Subsequent studies revealed that partial structures of LPS could inhibit LPS responses in human THP1 cells without inhibiting CD14-mediated

uptake of LPS, suggesting further, that molecules other than CD14 were important mediators of LPS signaling (Kitchens *et al.*, 1992). Nevertheless, a battery of studies emerged in 1993, providing evidence to support a role for CD14 in LPS signaling. It was shown that transfection of 70Z/3 cells (a murine pre-B cell line that does not express CD14), with human cDNA encoding mCD14 (hCD14), resulted in a one thousand-fold decrease in the concentration of LPS required to induce NF-kB translocation and mIgM expression (Lee *et al.*, 1992). In addition, tyrosine phosphorylation of p38 was detected in LPS-stimulated hCD14-transfected 70Z/3 cells (Han *et al.*, 1993). Transfection of hCD14 into Chinese Hamster Ovary (CHO) cells was shown to transfer macrophage-like LPS-responsiveness to an otherwise LPS-unresponsive cell line (Golenbock *et al.*, 1993). Transgenic mice, expressing high levels of CD14, were found to be hypersensitive to LPS (Ferrero *et al.*, 1993). By the end of 1993, the issue that CD14 may not be an absolute requirement for LPS signaling was revisited. Weinstein *et al.* (1993) reported that anti-CD14 antibodies did not prevent TP induced by high concentrations of LPS, suggesting the existence of CD14-dependent and CD14-independent components of a shared intracellular signaling pathway. Subsequently, anti-CD14 antibodies were identified that did not block LPS binding to CD14, but nonetheless, inhibited LPS -induced signaling (TP and NF-kB translocation), suggesting that CD14, itself, was not the transducer of LPS-induced signals (Lee *et al.*, 1993). In light of this, it was surprising that *src*-related kinase, *lyn*, co-immunoprecipitated with CD14 in LPS-stimulated human monocytes (described above; Stefanova *et al.*, 1993).

It is currently accepted that LPS stimulation utilizes both CD14-dependent and CD14-independent mechanisms. As a result, recent

investigations have been focused in two directions: (1) to dissociate CD14 dependent and CD14-independent pathways of intracellular signaling; and (2) to examine the physical nature of the LBP/LPS/CD14 complex in an attempt to define the interactive domains required for binding and signaling. Delude *et al.* (1994) found that CD14-mediated translocation of NF-κB in hCD14 transfected CHO cells was refractory to tyrosine kinase inhibitors (TKI); again, a surprising finding given the apparent association of CD14 with *lyn* in monocytes (above). Another study reported that neither anti-CD14 Ab nor TKI, affected LPS-induced activation of PKC, whereas both reagents suppressed the production of LPS-induced TNF-α (Shapira *et al.*, 1994), thus dissociating the function of CD14 from PKC activation. In sharp contrast, another study found that antibodies against CD14 blocked LPS-induced activation of PKC and TP of MAP kinases (Liu *et al.*, 1994). The discrepancies in these two studies may reflect different specificities of the antibodies used, as different antibodies against CD14 have been used to distinguish at least two functional domains in CD14; one that is responsible for LPS binding, and one that is required for signaling (Lee *et al.*, 1993; Gegner *et al.*, 1995).

sCD14 has been shown to mediate LPS-induced responses in cells that lack mCD14, such as human endothelial and epithelial cells (Frey *et al.*, 1992; Pugin *et al.*, 1993). Studies of LPS-induced TNF in the serum of patients suffering from paroxysmal nocturnal hemoglobinuria, a condition that results in impaired expression of GPI-linked membrane proteins (including CD14), indicated that low doses of LPS induced markedly reduced levels of TNF, whereas TNF levels in response to high concentrations of LPS were normal, further supporting a potential role for sCD14 (expressed normally in these patients) in mediating LPS responses (Duchow *et al.*, 1993). However,

using a combination of anti-CD14 antibodies and serum-free conditions, Lynn *et al.* (1993), detected LPS responses in human monocytes, and thus, concluded that neither CD14 nor serum (as a source for LBP, septin, and sCD14) is absolutely required for LPS stimulation.

Collectively, these data beg the question: What is the role of CD14? It appears that mCD14 mediates endotoxemia, as demonstrated by studies in which sCD14 prevented LPS-induced TNF production in human monocytes (Haziot *et al.*, 1994), and LPS-induced mortality in mice (Haziot *et al.*, 1995). Consistent with this result is the finding that LBP catalyzes the transfer of LPS from LPS/sCD14 complexes to HDL, thus implicating a role for sCD14 in LPS clearance (Wurfel *et al.*, 1995). However, increased levels of circulating sCD14 in septic patients has recently been correlated with increased mortality, suggesting that the beneficial aspects of sCD14 are subject to limitations (Landmann *et al.*, 1995). Interestingly, CD14 has recently been shown to mediate responses to polyuronic acid polymers, liboarabinomannan from mycobacteria, and cell wall preparations from gram positive bacteria, suggesting that CD14 may serve as a non-specific host defense mechanism by focusing a number of distinct bacterial components at the macrophage plasma membrane (Pugin *et al.*, 1994).

The above discussion revealed three interesting and apparently conflicting findings regarding CD14: (1) CD14 is GPI-linked and thus does not, itself, transduce the signals in response to LPS (Lee *et al.*, 1993); (2) *src*-related *lyn* kinase co-immunoprecipitates with CD14 in LPS-stimulated human monocytes (Stefanova *et al.*, 1993); and (3) CD14-mediated activation of NF-κB can be dissociated from tyrosine kinase activity (Delude *et al.*, 1994). At least two possibilities exist to account for these observations. First, GPI-anchored

receptor proteins may be linked to signaling molecules via lipid/lipid interactions. Secondly, perhaps another yet undefined membrane protein serves as the true "LPS signaling receptor" and associates with LPS-CD14 receptor complex upon binding of LPS, which would serve to stablize the complex and couple the interaction with *lyn*.

Several GPI-linked receptors have been shown to mediate cell signaling (e.g., Thy-t, Qa-2, CD14; reviewed in Robinson, 1991). In addition, receptor immunoprecipitates that contain *src*-related proteins, have also been shown to contain large quantities of glycolipids, suggesting the possibility that glycolipid microenvironments exist within membranes to couple GPI-linked receptors to signal transducing molecules (Casey, 1995). Signaling through certain receptors (e.g., Qa-2, a receptor on T cells), requires GPI-anchors, as transmembrane forms of the same receptors have been shown not to mediate cell activation (Robinson, 1991). In contrast, both GPI-anchored and integral membrane forms of CD14 have been shown to mediate cellular responses to endotoxin in 70Z/3 cells (Lee *et al.*, 1993), suggesting that the GPI tail is not important for LPS signaling. However, because sCD14 could not impart LPS-responsiveness to 70Z/3 cells, it was concluded that membrane expression of CD14 is required to initiate LPS signaling.

Using photoaffinity cross-linking techniques, a ~73 kDa LPS-binding protein was identified on the surface of lymphoreticular cell membranes, and mammalian peripheral blood monocytes (Lei and Morrison, 1988a; 1988b; Roeder *et al.*, 1989; Lei *et al.*, 1991; Halling *et al.*, 1992). Monoclonal antibodies against the ~73 kDa protein were generated (Bright *et al.*, 1990), and one of these antibodies (5D3), has been shown to mimic some of the characteristics of LPS. For example, 5D3 induces TNF production and synergizes with IFN-γ for

the production of $NO^{\bullet}$ and tumoricidal activity in *Lps*^{n}, but not *Lps*^{d} macrophages (Chen *et al.*, 1990; Green *et al.*, 1992). In addition, 5D3 was protective in mice against LPS-induced lethality (Morrison *et al.*, 1990) and thus, a very strong case has been presented to suggest a role for the 73 kDa LPS-binding protein in LPS signaling. However, a recent study has challenged the findings of Morrison *et al.*, claiming that the ~73 kDa protein also binds peptidoglycan and lipoteichic acid, and is, itself, cell-bound albumin (Dziarski, 1994). Interestingly, no data was provided to refute the LPS-mimetic effects observed with 5D3.

Rationale for the Work Presented Herein

The pupose of the introduction was to provide the historical and theoretical framework for the experiments carried out in my dissertation. Clearly, the LPS moiety of Gram negative bacteria elicits the involvement of an array of intracellular signaling pathways. However, how these pathways interact and culminate in gene expression has not been fully elucidated. I sought to further our understanding of the complex response to endotoxin by examining LPS-induced signaling events at three different levels: (1) at the nuclear level, with an analysis of a specific class of DNA binding proteins; (2) at the level of intracellular signaling, with an analysis of ser/thr phosphatase involvement in the signaling pathway leading to gene induction and TP; and, (3) at the plasma membrane level, through an analysis of the role of the ceramide pathway in LPS-induced signaling. Although the presentation and discussion of the data is organized to reflect regulation of LPS-induced gene expression at each of these levels, it is well appreciated that these levels are, in fact, inter-dependent upon one another and represent essential components of the coordinated macrophage response to LPS.

MATERIALS AND METHODS

Mice. C3H/OuJ, C3H/HeJ, and C57BL/6 mice (female, 5 weeks old) were obtained from the Jackson Laboratories (Bar Harbor, ME). Homozygous IRF-1 and IRF-2 "knock-out" (IRF-1$^{-/-}$ and IRF-2$^{-/-}$) mice (male and female; 5 - 14 weeks of age) were obtained directly from Amgen, Inc. (Thousand Oaks, CA). IRF-1$^{-/-}$ and IRF-2$^{-/-}$ mice had been inbred onto the C57BL/6 background for 6 generations. The mice were generally used within one week of receipt. Mice were maintained in a laminar flow facility under 12 hour light/dark cycles and fed autoclaved standard lab chow and acid water *ad libitum*.

Reagents. Protein-free (<0.008%), phenol-water-extracted *Escherichia coli* K235 lipopolysaccharide (LPS) was prepared by the method of McIntire *et al.* (1967). Protein-rich *S. minnesota* RaLPS was purchased from List Biologic Laboratories, Inc. (Campbell, CA). Protein-rich, butanol-extracted *Escherichia coli* K235 LPS was prepared by the method of Morrison and Leive, (1975). Natural murine IFN-α was purchased from Lee Biomolecular (San Diego, CA). Murine recombinant IFN-γ (rIFN-γ) was provided by Genentech, Inc. (South San Fransisco, CA). Cycloheximide (CHX) was purchased from Sigma Chemical Co. (St. Louis, MO). Calyculin A (GibcoBRL, Gaithersburg, MD) was dissolved in 10% dimethylsulfoxide (DMSO) as directed by the manufacturer. Three separate lots of calyculin A, as well as the DMSO, were tested for *Limulus* amoebocyte lysis activity (LAL) and found to contain <0.03 ng/ml LPS at the highest concentration used in these studies. Okadaic acid was purchased from Gibco BRL (Gaithersburg, MD). Two separate lots of okadaic acid were were tested for LAL and found to contain 0.1 ng/ml and <0.03 ng/ml LPS at the highest concentration used in these studies. C_2-ceramide (N-acetylsphingosine), C_6-ceramide (N-hexanoylsphingosine), and C_{16}-ceramide (N-palmitoylsphingosine)

were purchased from BIOMOL Research Laboratories, Inc. (Plymouth Meeting, PA) and dissolved either in DMSO or ethanol as directed by the manufacturer. Several lots of the ceramides were tested for LAL and all were found to contain <0.025 ng/ml LPS at the highest concentration used in these studies. Sphingomyelinase (*Staphylcoccus aureus)* was purchased from Sigma Chemical Co. (St. Louis, MO). Sphingomyelinase stocks varied in LAL activity and were found to contain between 1.1 - 12.0 ng/ml LPS.

Macrophage isolation and culture conditions. Peritoneal exudate macrophages were obtained by peritoneal lavage with sterile saline 4 days after intraperitoneal injection of 3 ml of sterile 3% fluid thioglycollate broth (BBL, Becton Dickinson Microbiology Systems, Cockeysville, MD). Cells were washed, resuspended in RPMI 1640 medium supplemented with 2 mM glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, 10 mM HEPES, 0.3% sodium bicarbonate, and 2% heat-inactivated (56° C, 30 min) fetal calf serum (FCS). Cells were added to 6-well tissue culture plates (Corning Glass Works, Corning NY) at ~4.5 x 10^6 cells per well in 2 ml or in 12-well tissue culture plates (Corning Glass Works) at ~2 x 10^6 cells per well in 1 ml. The plates were incubated at 37° C and 6% CO_2 for at least 4 h, but no longer than 18 h before the nonadherent cells were removed by washing with medium and the adherent cells were treated as indicated. This procedure routinely yields >95% of macrophages.

Induction of endotoxin tolerance *in vitro*. The protocol for establishing *in vitro* endotoxin tolerance has been described previously (Virca *et al.*, 1989). Peritoneal exudate cells were cultured (as above) for 4 to 6 h, after which non-adherent cells removed by washing twice with media. Adherent cells were treated with either medium or 100 ng/ml LPS and incubated for 19 h. The plates were then washed twice with excess media and the cells were re-cultured in media alone or 100 ng/ml LPS.

Isolation of Total Cellular RNA. Macrophages were cultured in 6-well plates with the indicated treatments, and lysed at the indicated time points with RNAzolB or RNA STAT-60 (1 ml/well; Tel-Test, Inc., Friendswood, Texas). Total cellular RNA was extracted according to the manufacturer's instructions and quantified by spectrophotometric analysis.

Oligonucleotide sequences. The primer sequences for IRF-1, IRF-2, IFN-β, and GAPDH were kindly provided by Dr. Carl Dieffenbach (NIAID, NIH). The primer sets for IRF-1 and IRF-2 correspond to the cDNA regions that exhibited minimal IRF sequence homology. The primer sets for IL-6, IL-10, iNOS, and p40 were provided by Dr. Cindy Salkowski (USUHS). The primer sequences for ICSBP were chosen using the computer-driven program "Primer Detective" (Clontech Labs, Inc., Palo Alto, CA), in conjunction with the published cDNA sequences obtained from GenBank. The primer sequences selected correspond to the cDNA region that exhibited the least homology with the other IRF family members. In addition, candidate primers were compared by computer alignment (GCG Gap Program) to IRF-1 and IRF-2 cDNA sequences to reduce the possibility of primer cross-reactivity. The predicted amplification product spans 3 introns. Table I lists the primer and probe sequences used for RT-PCR in this study.

Table I. Primer and probe sequences used for RT-PC

IRF-1 (5' → 3'):	Sense primer	CAGAGGAAAGAGAGAAAGTCC
	Antisense primer	CACACGGTGACAGTGCTGG
	Probe	GGACTCAGCAGCTCTACCCTA
IRF-2 (5' → 3'):	Sense primer	CAGTTGAGTCATCTTTGGGC
	Antisense primer	TGGTCATCACTCTCAGTGG
	Probe	TTCTCCTGAGTATGCGGTCC
IFN-β (5' → 3'):	Sense primer	AGATCAACCTCACCTACAGG
	Antisense primer	TGGAGTTCATCCAGGAGAC
	Probe	CCATCCAAGAGATGCTCCAG
GAPDH (5' → 3'):	Sense primer	CCATGGAGAAGGCTGGGG
	Antisense primer	CAAAGTTGTCATGGATGACC
	Probe	CTAAGCATGTGGTGGTGCA
ICSBP (5' → 3'):	Sense primer	GATCAAGGAACCTTCTGTGG
	Antisense primer	GAAGCTGATGACCATCTGGG
	Probe	ATGAGTACATGGGTATGACC
IL-6 (5' → 3'):	Sense primer	TTCCATCCAGTTGCCTTCTTGG
	Antisense primer	CTTCATGTACTCCAGGTAG
	Probe	ACTTCACAAGTCCGGAGA
IL-10 (5' → 3'):	Sense primer	CGGGAAGACAATAACTG
	Antisense primer	CATTTCCGATAAGGCTTGG
	Probe	GGACTGCCTTCAGCCAGGTGAAGACTTT
iNOS (5' → 3'):	Sense primer	CCCTTCCGAAGTTTCTGGCAGCAGC
	Antisense primer	GGCTGTCAGAGCCTCGTGGCTTTGG
	Probe	CAAGGTCTACGTTCAGGACATC
p40 (5' → 3'):	Sense primer	CGTGCTCATGGCTGGTGCAAAG
	Antisense primer	GAACACATGCCCACTTGCTG
	Probe	GCTCAGGATCGCTATTAC

RT-PCR. cDNA synthesis from 1 μg or 2 μg aliquots of total cellular RNA was carried out using 100 ng of specific antisense primers or random hexamers, respectively. RNA and primers were mixed with reverse transcriptase buffer (50 mM Tris-HCl, pH 8.3, 75 mM KCl, 10 mM DTT, 0.3 mM $MgCl_2$), and 0.5 mM of each deoxyribonucleoside triphosphate (Pharmacia, Piscataway, NJ) in a final volume of 20 μl. After an initial incubation at 70°C for 5 min, the tubes were placed on ice for 5 min after which 200 U of M-MLV RT reverse transcriptase (BRL, Bethesda, MD) was added to each reaction tube. The tubes were incubated at 37°C for 30 min followed by 10 min of heating at 90° C. One and one-half to 3 μl of cDNA was added to tubes containing 100 ng of specific sense and antisense oligonucleotide primers (see below), Taq buffer (500 mM KCl; 100 mM Tris-HCl; 1% Triton X-100), 3 mM $MgCl_2$, 200 mg dXTP, and 1 U Amplitaq (Cetus, Emeryville, CA) DNA polymerase. PCR cycling was performed in an automated DNA Thermal Cycler Model 480 (Perkin Elmer Cetus Instrumentation, Norwalk, CN), with each cycle consisting of 1 min denaturing at 94° C, 1 min annealing at 54 - 57° C, and 1 min extension at 72° C, following an initial 3 min incubation at 94° C. A 25 μl sample of amplification product was electrophoresed in TBE through a 2% agarose gel containing 0.3 mg/ml ethidium bromide, after which the gel was denatured (1.5 M NaCl; 0.5 N NaOH) for 20 min, neutralized (0.5 M Tris-HCl; 1.5 M NaCl) for 10 min, and the DNA was transferred to Nytran (Schleicher & Schuell, Keene, NH) by capillary action according to standard Southern blotting protocol (Southern, 1975). The membranes were UV-crosslinked in a Stratalinker 1800 (Stratagene, La Jolla, CA), vacuum-baked at 60°C for 1 h, and hybridized to a specific internal oligonucleotide probe. The probes were labeled and detected using the enhanced chemiluminescence (ECL) protocol according to the manufacturer's instructions (Amersham, Arlington Heights,

IL). The Kodak X-omat AR 5 film used to detect the light emission, was subjected to densitometric analysis on a datacopy GS plus scanner (Xerox Imaging systems, Sunnyvale, CA), after which the individual band signals were measured using the NIH Image 1.42b16 program. In order to determine optimal PCR conditions, the input RNA was held constant and PCR amplification products were sampled over a range of cycle numbers. A plot of cycle number *vs*. PCR signal was used to select the optimal cycle number within the linear range of signal detection. In addition, serial two-fold dilutions of input RNA were analyzed by RT-PCR at the optimal cycle number, which established a linear relationship between input RNA and PCR signal, thus demonstrating the ability of this technique to detect at least two-fold differences of initial mRNA copy number. In order to quantify changes in mRNA levels, serial two-fold dilutions of PCR amplification products (known to be positive for the gene of interest) were subjected to ECL analysis for each experiment, in order to relate changes in product concentration to changes in ECL signal. Linear regression of each standard curve generated an equation of the line to be used in the calculation of relative gene expression in the samples of interest. In addition, each sample was normalized to the expression of the "housekeeping" gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH), which was not modulated by any of the treatments used. All computer-generated images of the scanned ECL blots were reproduced with Aldus FreeHand 3.11 software.

Probes used for Northern blot analysis. The probes used in these experiments were as follows: ICSBP cDNA was a 1-kb *Eco* RI insert from plasmid 131A (Driggers *et al.*, 1990); β-actin cDNA was a 0.75-kb *Eco*RI insert (Tokunaga *et al.*, 1986); 1100 bp segment of the TNF-α cDNA (Pennica et al., 1985); 275 - 1329

segment of IL-1β cDNA (Ohmori *et al.*, 1990); 450 bp segment of IP-10 cDNA (Ohmori and Hamilton, 1990); D7 (TNFR-2) cDNA (Tannenbaum et al., 1988). The IRF-1 and IRF-2 DNA probes were prepared using the TA Cloning System Version 1.3 of Invitrogen Corporation (San Diego, CA) with the following specifications or modifications: RT-PCR was performed as outlined above except that the extension time for the final cycle was increased to 7 min. Single DNA bands of the correct MW were excised from the gel and subjected to Gene Clean (BIO 101, La Jolla, CA) purification followed by spectrophotometric analysis of yield. Approximately, 140 - 160 ng of purified IRF-1 or IRF-2 PCR-derived DNA was precipitated with 75 ng cloning vector (provided in the kit) in 0.3 M NaOAc (pH 7-7.5) and 3 volumes of ice cold ethanol overnight at 4° C. The DNA was collected by centrifugation, washed with 75% ethanol, and resuspended in 9 µl of sterile water to which 1 µl of ligation buffer (provided in the kit) and 1 µl of T4 DNA ligase (provided in the kit) were added followed by an overnight incubation at 12° C. Following the transformation procedure provided, white colonies were replica plated onto Whatman nitrocellulose filters (Whatman Inc., Clifton, NJ) that were overlaid onto antibiotic selective media and incubated overnight at 37°C. The filters were removed, denatured (as described above), neutralized (as described above), washed with sterile water, and probed with specific internal oligonucletides that were end-labeled with [γ-^{32}P]dATP using T4 polynucleotide kinase (Gibco, Grand Island, NY). Plasmids from the transformants were subsequently extracted using Circle Prep (BIO 101, La Jolla, CA) and subjected to restriction enzyme digestion, electrophoresis and oligonucleotide probe hybridization to confirm the integrity and specificity of the clones. Each clone was sequenced using the Applied Biosystems Taq Dye-deoxy Terminator Cycle Sequencing protocol with the following

specifications: Isolation of the plasmids carrying the IRF-1 or IRF-2 cDNA was carried out using the Qiagen plasmid mini-prep system (QIAGEN Inc., Chatsworth, CA) according to the manufacturer's instructions. The vector-specific primers used were SP6 and T7. Cycling was carried out on a DNA Thermal Cycler model 480 (Perkin Elmer Cetus Instrumentation, Norwalk,CN). Extension products were separated from unincorporated dye-deoxynucleosides using Centri-Sep columns (Princeton Separations, Inc., Adelphia, N.J.) as directed by the manufacturer. Termination products were separated by agarose gel electrophoresis and the corresponding sequences were determined using Applied Biosystems Model 373A Automated DNA sequencer. The IRF-1 clone contains nucleotides 357-564 of the published cDNA sequence (Miyamoto *et al.*, 1988). The IRF-2 clone contains nucleotides 421-635 of the published cDNA sequence with a 6 bp deletion of nucleotides 531-536 (Harada *et al.*, 1989). The GAPDH probe contains 195 bp of GAPDH cDNA (Fort *et al.*,1985) and was prepared as described above.

Northern blot analysis. Ten μg of total cellular RNA was denatured by heating at 90° C for 5 min in 1.5X loading buffer (Davis, L. G., 1986), quickly cooled on ice then electrophoresed in 1X MOPS through a 1.5% agarose gel containing 0.66 M formaldehyde and 0.3 mg/ml ethidium bromide. Following photography, the gel was rinsed in 10X SSC and the RNA was transferred to Nytran (Schleicher and Schuell, Keene, NH.) overnight by capillary action. The blots were then UV-crosslinked and stored in pre-hybridization fluid (Davis, L. G., 1986) at -20° C until probed. Fifty-100 ng of the double stranded probes were labeled with [α-^{32}P]dCTP using a random primer oligolabeling kit (Pharmacia, Piscataway, N.J.) Phosphor screens were exposed to probed blots and the band intensity was quantified using

PhosphorImager model 400A and Image Quant 3.0 software (Molecular Dynamics Inc., Sunnyvale, CA). Kodak X-omat AR 5 film was also exposed to the probed blots, and was scanned as described above. All computer-generated images of the scanned Northern blots were reproduced with Aldus FreeHand 3.11 software.

Phosphatase Assay. Macrophages were cultured in 12-well plates, treated as indicated, and washed with 2% RPMI to remove any remaining extracellular calyculin A. Whole cell lysates were prepared by adding 240 µl/well of ice cold lysis buffer [50 mM Tris-HCl, pH 7.0 at 25° C; 0.1 mM EDTA; 0.1 mM EGTA; 0.1% (v/v) β-mercaptoethanol; 25 µg/ml leupeptin; 25 µg/ml aprotinin; 0.5% (v/v) Triton X-100]. The plates were incubated on ice and placed on a mini-orbital shaker (Bellco Glass Inc., Vineland, NJ) for 10 min on setting 3. The lysates were then pipetted up and down, transferred to 1.5 ml microcentrifuge tubes, and centrifuged at 12,000 X *g* (Eppendorf model 5415C) for 5 min. The supernatants were collected and either used immediately or stored at -70° C. Phosphatase activity was determined using the Protein Phosphatase Assay System (GibcoBRL, Gaithersburg, MD), according to the manufacturer's instructions. A Beckman LS7500 scintillation counter (Beckman Instruments, Inc., Fullerton, CA) was used to quantify the counts per minute (cpm).

Measurements of $NO^{\bullet}$. Secretion of $NO^{\bullet}$ by macrophages was determined by measuring nitrite as decribed elsewhere (Lorsbach *et al.*, 1993). Supernatants were mixed with an equal volume of Griess reagent (1 part 0.1% N-[napthyl] ethylenediamine dihydrochloride in H_2O and 1 part 1% sulfanilamide in 5% H_3PO_4). Absorbance at 570 nm was measured and compared to a $NaNO_2$ standard curve.

Bioassay to detect TNF activity. To measure TNF activity, a standard TNF cytotoxicity bioassay was performed as previously described (Hogan and Vogel, 1990).

Monolayers of L929 fibroblasts were prepared by overnight incubation of 4×10^4 cells/well in 96-well micotiter plates (Falcon Plastics, Oxnard, CA). The medium was aspirated from each well and replaced with 50 µl fresh medium and 50 µl of the test sample. Ten serial two-fold dilutions were then made and 50 µl actinomycin D (8 mg/ml; Sigma Chemical Co., St. Louis MO) was added per well. The plates were incubated for 18 h at 37° C and 6% $C0_2$, followed by washing with normal saline and staining with 0.05% crystal violet in 20% ethanol for 1 h. The plates were then rinsed in tap water and air dried after which the stain was eluted with 100 µl absolute methanol and the 595 nm absorbance was read in a Biotek EL 380 microtiter plate reader (Biotek Instruments, Inc., Winooski, VT). TNF activity in units/ml represents the reciprocol of the highest dilution that resulted in 50% cell death, multiplied by the dilution factor. In some experiments, TNF-α concentrations were determined using a commercially available murine TNF-α ELISA kit (Genzyme, Cambridge, MA).

Bioassay to detect IFN activity. To measure IFN activity, a standard antiviral assay was performed as previously described (Vogel and Hogan, 1990). Test samples (50 µl/well) were serially diluted into microtiter plates as described above. L929 fibroblasts were added (1×10^5 cells/well) in a volume of 50 µl and the plates were incubated for 24 h at 37° C and 6% CO_2. The supernatants were aspirated and 100 µl vesicular stomatitis virus (Indiana strain; multiplicity of infection = 0.1) was added per well in medium containing 10% FCS. Following 18-24 h of infection, the wells were washed three times with Earle's balanced salt solution and fixed with 5% formalin for 10 min. The cells were stained (30 min), washed, and dried as described above. Stain was eluted as described above, prior to reading the 595 nm absorbance. The endpoint of the assay was defined as the first well within a serial dilution which exhibited an optical density equal to that of a virus control well (i.e., containing only

L929 cells and virus). Units/ml represent the reciprocal titers based on a titration of 100 U/ml National Institutes of Health (NIH) mouse fibroblast IFN reference standard (G-022-904-511; Research Resources Branch, National Institute of Allergy and Infectious Diseases).

Anti-phosphotyrosine and anti-MAP kinase immunoblotting. The preparation of antiphosphotyrosine immunoblots has been detailed elsewhere (Manthey *et al.*, 1993). Macrophages were cultured in 12-well plates with the indicated treatments and lysed with 200 µl/well of ice cold lysis buffer (100 mM Tris-HCl, pH 8.0; 100mM NaCl; 2 mM EDTA; 1% NP-40; 1 mM Na_3VO_4; 50 mM NaF; 100 µM TPCK; 100 mM quercitin; 1 mM PMSF; 1 µg/ml leupeptin and pepstatin). The plates were incubated in an ice bath and agitated on a mini-orbital shaker as described above. Cell lysates were transferred to microfuge tubes and centrifuged 12,000 X *g* for 1 min. Supernatants (160 µl) were boiled for 5 min with 56 µl/tube 4X loading buffer (200 mM Tris-HCl, pH 6.8; 10% SDS; 400 mM DTT; 40% glycerol; 0.4% bromophenol blue) and 13 µl/lane was separated by SDS-PAGE on 10% acrylamide gels (7 x 8 cm) using a 375 mM Tris-HCl (pH 8.5) running buffer. Following transfer onto Immobilon-P transfer membranes (Millipore Corp., Bedford, MA), blots were blocked for 1 h in buffer (20 mM Tris-HCl; pH 7.6; 137 mM NaCl; and 0.1% Tween 20) containing 3% gelatin and 5% milk and then incubated for 1 h in buffer that contained 1 µg/ml antiphosphotyrosine mAb (4G10) or polyclonal antibodies specific for MAP kinases *erk-1* and *erk-2* (both purchased from Upstate Biotechnology, Inc., Lake Placid, NY). The blots were then washed and incubated with horseradish peroxidase-conjugated goat anti-mouse IgG (for antiphosphotyrosine; Bio-Rad Laboratories, Richmond, CA) or goat anti-rabbit IgG (for anti-*erk-1* and *erk-2*; Bio-Rad

Laboratories) for 1 h. The enhanced chemiluminescence (ECL) detection method (Amersham, Amersham, UK) was used to visualize the signals. Molecular weight determinations were based on comparisons with prestained markers (Bio-Rad Laboratories).

RESULTS

ANALYSIS OF THE POTENTIAL ROLE OF IRF FAMILY MEMBERS IN LPS-INDUCED SIGNALING LEADING TO GENE EXPRESSION

As presented in the Introduction, the nuclear transcription factors that are known to participate in LPS-signaling include NF-κB, *jun*, CREB, PU.1, Elk-1 and SAP-1. When I initiated these studies, I sought to identify additional transcription factors that may regulate LPS-inducible gene expression in macrophages. The existence of two LPS-hyporesponsive models provided the direction and ideal background with which to examine the regulation of potential LPS-inducible transcription factors. In both the C3H/HeJ (*Lps*^{d})-derived macrophages, and the C3H/OuJ (*Lps*^{n})-derived macrophages rendered LPS "tolerant", the hyporesponsive state is characterized by low IFN production in response to LPS (reviewed in Vogel, 1992; Virca *et al.*, 1989).

The transcriptional regulation of IFN and IFN-inducible gene expression has been an area of active research that has led to the identification of a novel family of DNA binding proteins, referred to as "Interferon Regulatory Factors" (IRFs) (Miyamoto *et al.*, 1988). There are presently four members of the IRF family: IRF-1 (ISGF2), IRF-2 (ISGF1), interferon consensus sequence binding protein (ICSBP), and the γ subunit of interferon gene factor 3 (ISGF3γ) based on the high degree of homology in the DNA binding domains of these proteins (Driggers *et al.*, 1990; Harada *et al.*, 1989; Miyamoto *et al.*, 1988; Pine *et al.*, 1990; Veals *et al.*, 1992). IRF-1, IRF-2, and ICSBP bind to the IRF DNA recognition sequence, (G/C)(A)AAA(N)$_{2-3}$AAA(G/C)(T/C), that is present in the 5' flanking regions of the IFN genes and in the interferon-

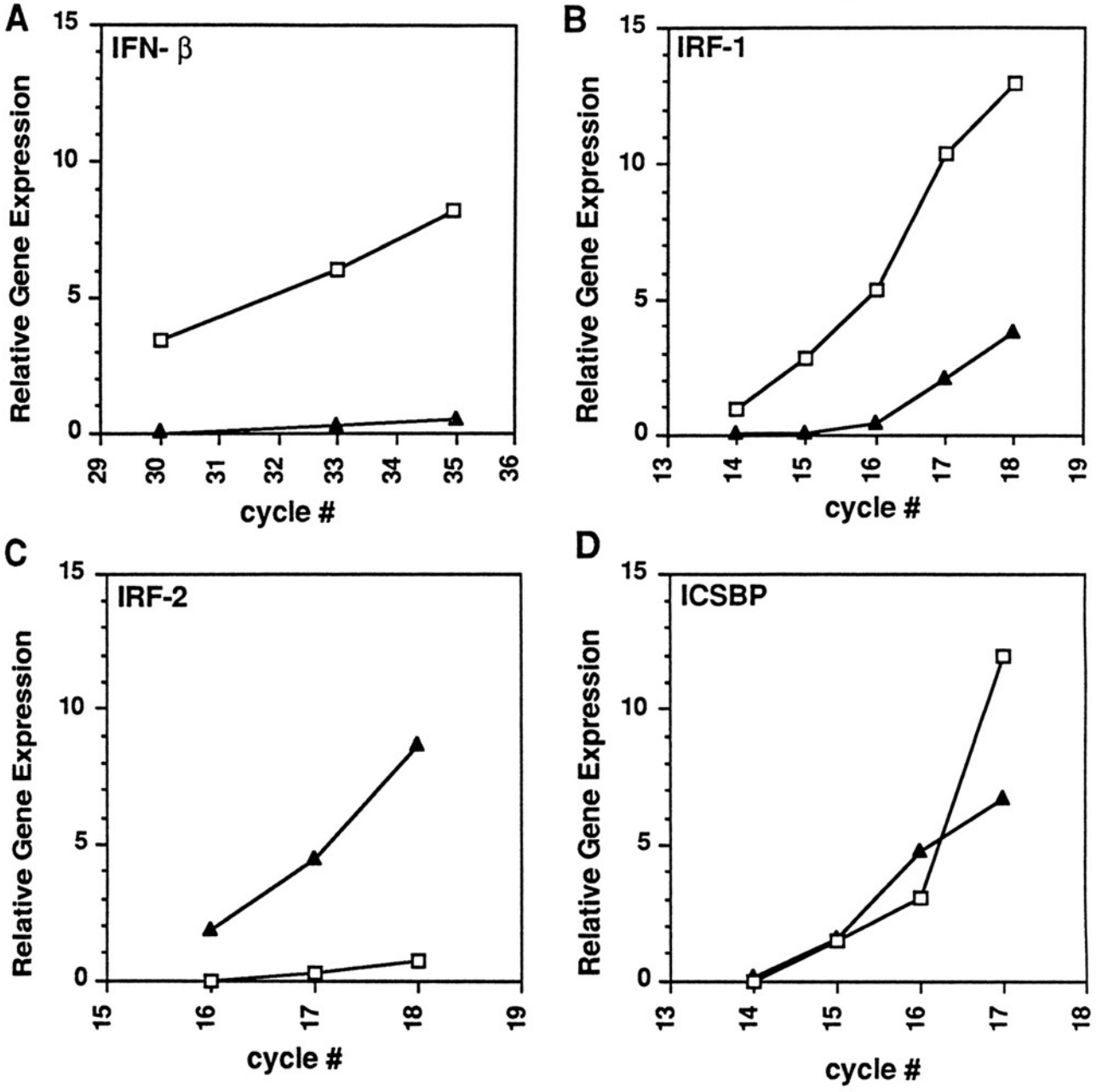
stimulated response elements (ISRE) of many IFN-inducible genes (Tanaka *et al.*, 1993; Driggers *et al.*, 1990). In contrast, ISGF3γ binds only weakly to DNA when not complexed with ISGF3α (not an IRF family member), and does not bind to the type I IFN promoter elements recognized by the other IRFs, due to its requirement for additional DNA sequences provided by the ISRE (Kessler *et al.*, 1988; Kessler *et al.*, 1990; Veals *et al.*, 1991). The highly defined function of ISGF3γ in IFN-induced signal transduction has been well described (Veals *et al.*, 1993). Transfection studies have suggested that IRF-1 functions as a transcriptional activator, while IRF-2 and ICSBP serve to repress IRF-1-induced transcription (Harada *et al.*, 1989; Nelson *et al.*, 1993). In addition, it has been suggested that an increase in the ratio of IRF-1/IRF-2 in the cell may be a critical event in IRF-1 mediated transcription (Tanaka *et al.*, 1993); however, the quantification of available IRF-1 and IRF-2 in a cell is undoubtedly more complicated in light of the recent finding that ICSBP forms protein complexes with the other three IRF family members (Bovolenta *et al.*, 1994). Thus, the dynamic interactions between the individual IRF proteins and their common target DNA sequence may determine the pattern of gene expression that results in a responsive or hyporesponsive phenotype.

Therefore, the potential involvement of the IRFs in LPS signaling was examined. To this end, LPS-inducibility of IRF-1, IRF-2, and ICSBP mRNA and the relative gene expression of these transcription factors in LPS-responsive and LPS-hyporesponsive macrophages were compared. In addition, the availability of mice engineered with targeted disruptions of either the IRF-1 gene (IRF-1$^{-/-}$) or the IRF-2 gene (IRF-2$^{-/-}$; Matsuyama *et al.*, 1993) enabled us to assess the roles of IRF-1 and IRF-2 genes by comparing

LPS-inducible gene expression in macrophages derived from control C57BL/6, IRF-1$^{-/-}$, and IRF-2$^{-/-}$ mice.

Differential basal expression of IRF-1, IRF-2, and IFN-β steady-state mRNA levels in *Lps^n versus Lps^d* macrophages. To compare IFN-β and IRF family gene expression in macrophages that exhibit the genetically determined, LPS-responsive or LPS-hyporesponsive phenotypes, total cellular RNA was extracted from peritoneal exudate macrophages derived from C3H/OuJ (*Lps^n*) or C3H/HeJ (*Lps^d*) mice. RNA preparations were subjected to RT-PCR and ECL signal detection with primers and probes specific for IRF-1, IRF-2, ICSBP, IFN-β, or GAPDH mRNA sequences. Detection of IFN-β (Figure 1A) and IRF-1 (Figure 1B) mRNA in *Lps^n* macrophages required fewer cycles of amplification than required to detect IFN-β and IRF-1 mRNA in *Lps^d* macrophages. Comparison of relative gene expression at the first cycle number where both samples have detectable IFN-β and IRF-1 (cycle #33 and 16, respectively) reveals ~15 - 22 fold higher levels of steady-state IFN-β and IRF-1 mRNA in *Lps^n* macrophages than in *Lps^d* macrophages. In contrast, mRNA specific for IRF-2 (Figure 1C) is detected in *Lps^d* macrophages after fewer cycles of amplification (cycle #16) than that required to detect IRF-2 mRNA in *Lps^n* macrophages (cycle #17). Comparison of relative gene expression at the first common cycle number (cycle #17) demonstrates that *Lps^d* macrophages contain ~18-fold higher levels of steady-state IRF-2 mRNA than *Lps^n* macrophages. There was no consistent differential expression of ICSBP mRNA between the two strains (Figure 1D).

Figure 1. Basal level expression of IFN-β, IRF-1, IRF-2, and ICSBP mRNA in C3H/OuJ and C3H/HeJ macrophages. Peritoneal exudate macrophages from C3H/OuJ (□) and C3H/HeJ (▲) mice were cultured as described in the Materials and Methods. RNA extracted from untreated macrophages was subjected to RT-PCR over the indicated cycle numbers. Basal levels of IFN-β, IRF-1, IRF-2, and ICSBP mRNA are shown in Figure 1A, B, C, and D, respectively. The data has been normalized to GAPDH gene expression ("Relative Gene Expression") as described in Materials and Methods and are derived from a single experiment that is representative of at least 3 independent experiments.


A
IFN- β
Relative Gene Expression
cycle #
B
IRF-1
Relative Gene Expression
cycle #
C
IRF-2
Relative Gene Expression
cycle #
D
ICSBP
Relative Gene Expression
cycle #

***Lps^d* macrophages respond to IFN-α but not to LPS to modulate IRF gene expression.** The experiment depicted in Figure 1 associated the molecular phenotype of C3H/HeJ macrophages with higher basal levels of IRF-2 mRNA than C3H/OuJ macrophages. To address the possibility that exposure to LPS somehow promotes elevated expression of transcriptional repressors in *Lps^d* macrophages, we compared IRF-1, IRF-2, and ICSBP mRNA levels in C3H/HeJ and C3H/OuJ macrophages stimulated with 100 ng/ml LPS. In addition, since many of the functional deficiencies in macrophage differentiation associated with expression of the *Lps^d* allele are reversible by the addition of exogenous IFN (Fertsch *et al.*, 1984), the pattern of IRF gene expression in *Lps^d* macrophages stimulated with 500 U/ml IFN-α was also analyzed. Total cellular RNA was extracted at various times over 10 h and subjected to Northern blot or RT-PCR analysis. In contrast to *Lps^n* macrophages (Figure 2), there was no measurable modulation of IRF-1, IRF-2, or ICSBP mRNA following LPS stimulation of *Lps^d* macrophages (Figure 3). However, *Lps^d* macrophages did respond to IFN-α stimulation (Figure 4). IRF-1 mRNA levels increased rapidly, reaching maximal levels of ~70 fold by 4 hours. IRF-2 mRNA levels increased later and to a lesser extent (~10 fold), also peaking by 4 hours. Consistent with previous findings (Driggers *et al.*, 1990; Politis *et al.*, 1994), ICSBP mRNA was not notably modulated by IFN-α.

Cycloheximide does not inhibit the accumulation of LPS-induced IRF-1 mRNA. Since IFN and other LPS-inducible cytokines have been reported to induce IRF family gene expression (Miyamoto *et al.*, 1988; Harada *et al.*, 1989; Fujita *et al.*, 1989; Driggers *et al.*, 1990; Pine *et al.*, 1990), it was of interest to determine if induction of these genes was a direct or indirect result of LPS stimulation. To address this point, *Lps^n* macrophages were treated with

Figure 2. IRF-1, IRF-2, ICSBP, and β-actin gene expression in C3H/OuJ macrophages treated with LPS. Macrophage cultures were treated with medium or 100 ng/ml LPS. RNA was extracted at the indicated times after treatment and subjected to Northern blot analysis. The data shown is derived from a single experiment that is representative of at least 3 independent experiments.

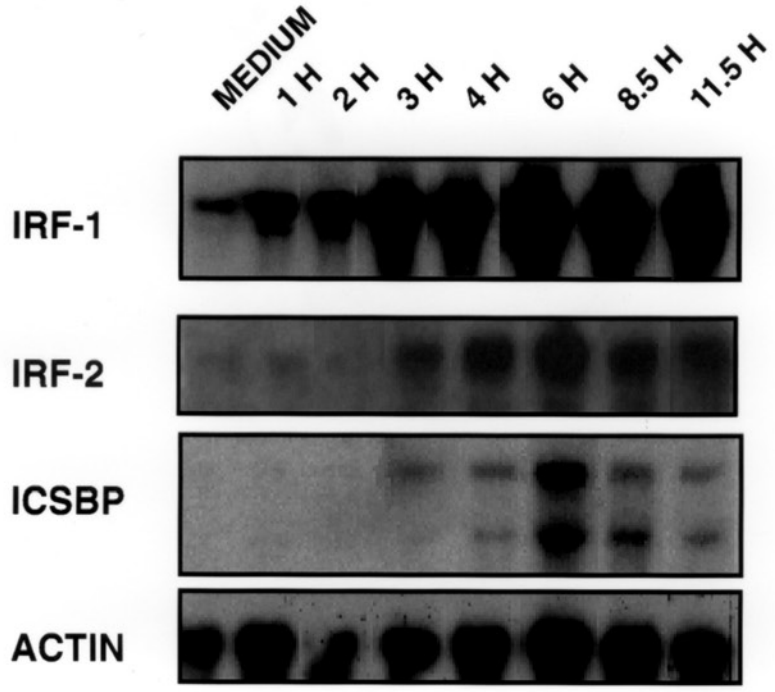

MEDIUM
1 H
2 H
3 H
4 H
6 H
8.5 H
11.5 H
IRF-1
IRF-2
ICSBP
ACTIN

Figure 3. IRF-1, IRF-2, ICSBP, and β-actin gene expression in C3H/HeJ macrophages treated with LPS. Macrophages were treated with medium or 100 ng/ml LPS. RNA was extracted at the indicated times after treatment and subjected to Northern blot analysis. The data shown is derived from a single experiment that is representative of at least 3 independent experiments.

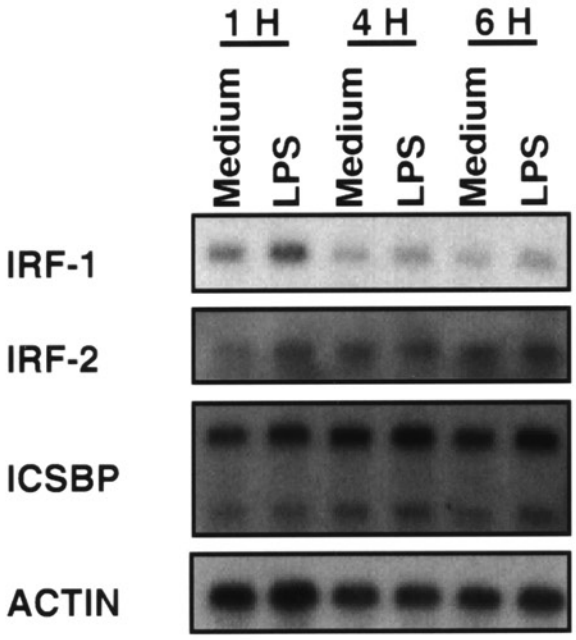
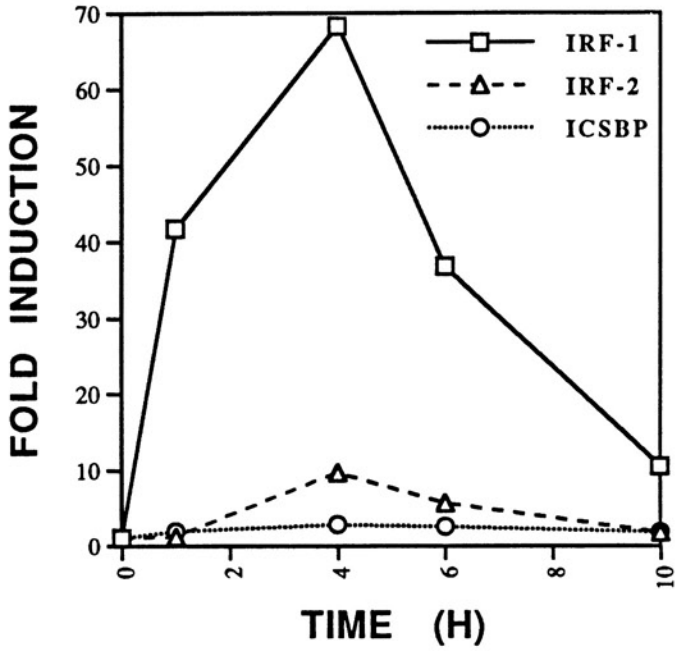

1 H
4 H
6 H
Medium
LPS
Medium
LPS
Medium
LPS
IRF-1
IRF-2
ICSBP
ACTIN

Figure 4. IRF-1, IRF-2, and ICSBP gene expression in C3H/HeJ macrophages treated with IFN-α. Macrophages were treated with medium or 500 U/ml IFN-α. RNA was extracted at the indicated times and subjected to RT-PCR analysis for 24 cycles. The data shown has been normalized to GAPDH gene expression, as described in Materials and Methods, and is derived from a single experiment that is representative of at least 3 independent experiments.


FOLD INDUCTION
70
60
50
40
30
20
10
0
0
2
4
6
8
10
TIME (H)
IRF-1
IRF-2
ICSBP

medium or 100 ng/ml LPS, in the absence or presence of 5 μg/ml cycloheximide. Total RNA was extracted after 4.5 hours and subjected to RT-PCR. The results, shown in Figure 5, indicate that LPS, in the presence of cycloheximide, results in superinduction of IRF-1 mRNA. In contrast, accumulation of LPS-induced IRF-2 and ICSBP mRNA is abolished in the presence of cycloheximide.

LPS stimulation of endotoxin-tolerized macrophages does not result in an increase of IRF-1, IRF-2, or ICSBP steady-state mRNA levels. Prior exposure of macrophages to LPS results in a transient state of LPS-hyporesponsiveness (Virca *et al.*, 1989), during which certain LPS-inducible genes are refractory to re-induction upon LPS re-stimulation (Henricson *et al.*, 1993). Since C3H/HeJ LPS-hyporesponsive macrophages exhibited higher basal levels of IRF-2 mRNA than *Lps*^{n} macrophages, and since IRF-2 has been shown to exert transcriptional repressor activity (Harada *et al.*, 1989), *Lps*^{n} macrophages rendered endotoxin tolerant *in vitro* were also assessed for IRF gene expression. Figure 6 illustrates gene expression in tolerized macrophages that were re-stimulated with either medium or 100 ng/ml LPS for 1 or 4 h. For each gene examined, the level of restimulated mRNA is shown relative to basal levels (MEDIUM/MEDIUM; macrophages pre-treated with medium and "challenged" with medium) and maximally inducible levels (MEDIUM/LPS; macrophages pre-treated with medium and stimulated with LPS for 4 h). At 1 h after re-stimulation with medium or LPS, levels of IRF-1 and ICSBP mRNA were still near the maximally LPS-inducible levels measured in the experiment. (In 3 separate experiments, IRF-1 and ICSBP gene expression in LPS/MEDIUM samples was 75-95% of that measured in the maximally induced MEDIUM/LPS samples.) In contrast, IRF-2 mRNA was expressed in

Figure 5. Effect of cycloheximide (CHX) on LPS-inducible IRF-1, IRF-2, and ICSBP gene expression. C3H/OuJ macrophage cultures were treated with media, 100 ng/ml LPS, 5 μg/ml CHX, or 5 μg/ml CHX plus 100 ng/ml LPS. RNA was extracted at 4.5 hours after treatment and subjected to RT-PCR for 20 cycles. The data shown is derived from a single experiment that is representative of at least 3 independent experiments and has been normalized to GAPDH gene expression ("Relative Gene Expression") as described in Materials and Methods.

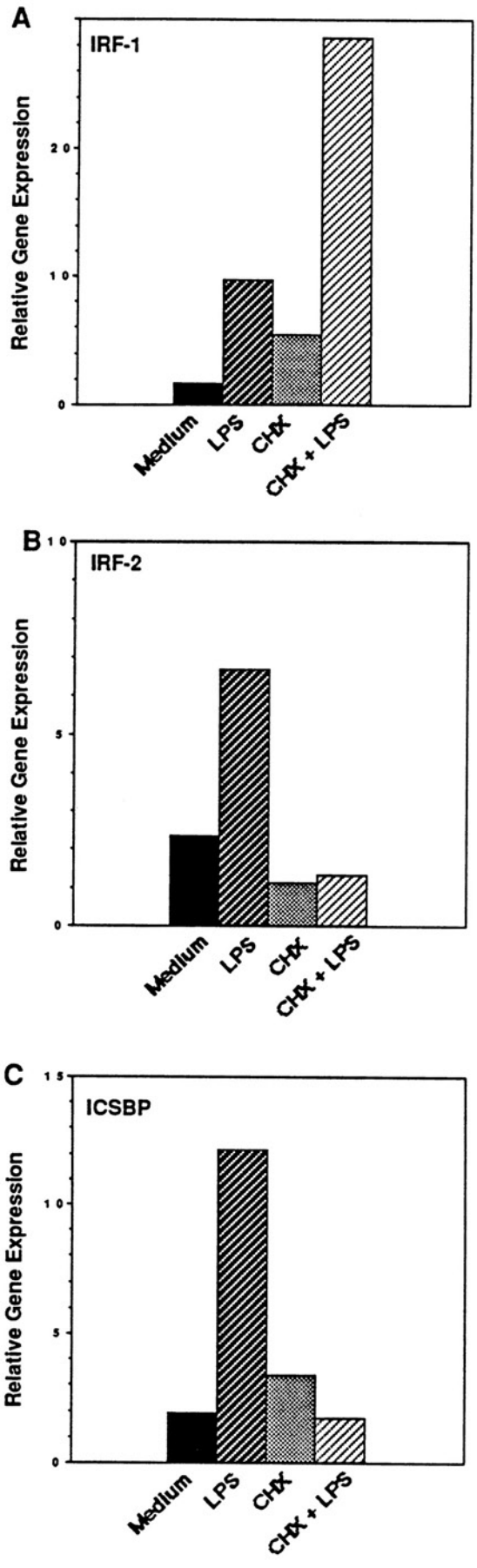
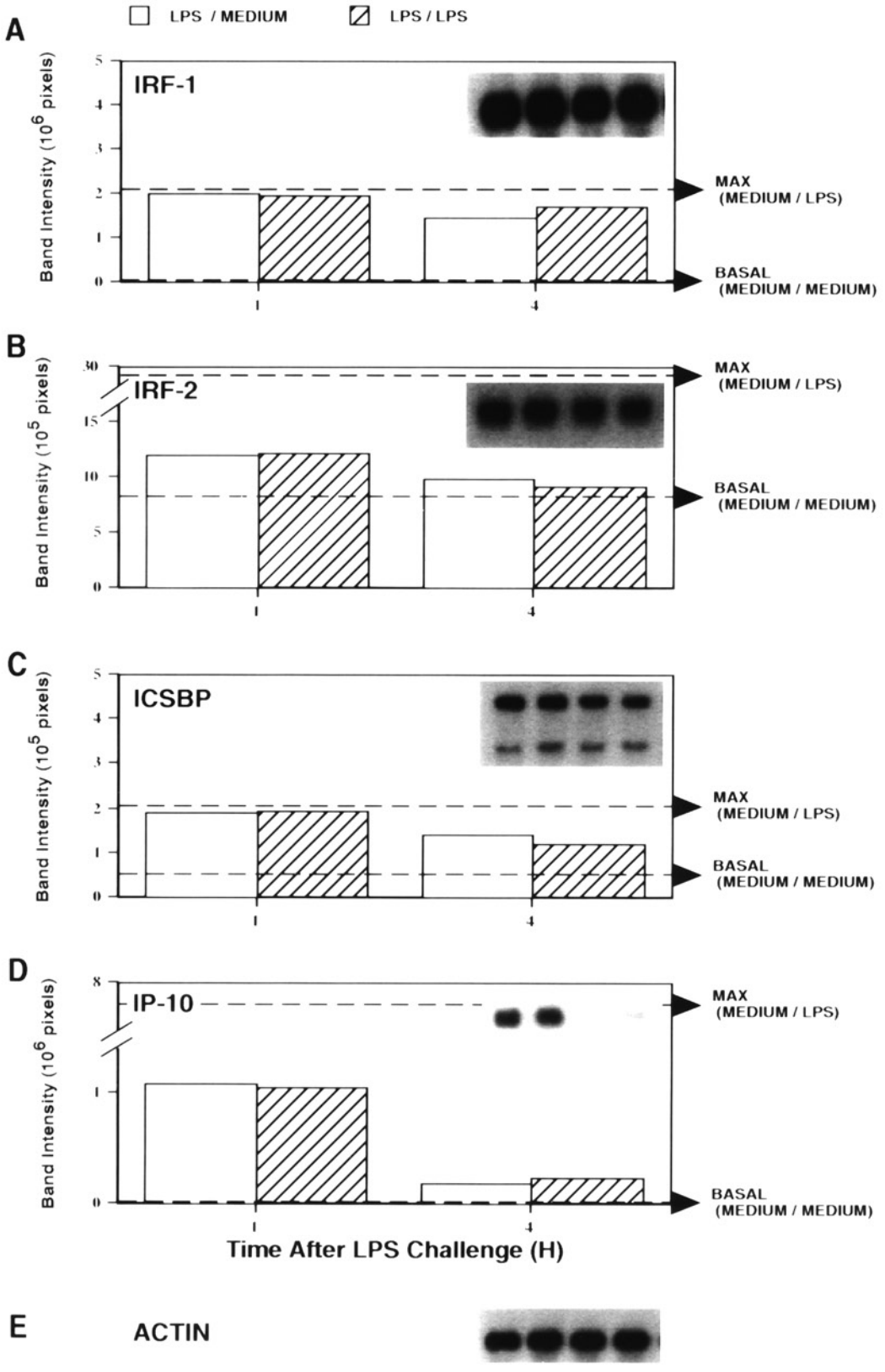

A
IRF-1
Relative Gene Expression
0
10
20
Medium
LPS
CHX
CHX + LPS
B
IRF-2
Relative Gene Expression
0
5
10
Medium
LPS
CHX
CHX + LPS
C
ICSBP
Relative Gene Expression
0
5
10
15
Medium
LPS
CHX
CHX + LPS

Figure 6. IRF-1, IRF-2, ICSBP, and IP-10 gene expression in endotoxin-tolerized C3H/OuJ macrophages. C3H/OuJ macrophages were pre-treated with medium or 100 ng/ml LPS. After 19 h, the cells were washed twice with medium and "challenged" with either medium or 100 ng/ml LPS. RNA was extracted at the indicated times and subjected to Northern blot analysis. Treatment codes are expressed as pre-treatment/treatment. LPS/MEDIUM (open) and LPS/LPS (hatched) represent tolerized cells re-stimulated with medium or LPS, respectively. Basal levels are indicated by a dashed line and represent gene expression in the cells treated with medium at both times (MEDIUM/MEDIUM). The maximal LPS-inducible gene expression (MEDIUM/LPS) measured in the experiment is also indicated by a dashed line. The inset autoradiograms for each gene show the Northern blot results of LPS/MEDIUM, LPS/LPS, LPS/MEDIUM, and LPS/LPS, respectively, from left to right. IRF-1, IRF-2, ICSBP, IP-10, and β-actin gene expression data, from a single experiment that is representative of at least 3 independent experiments, is shown in Figure 6A, B, C, D, and E respectively.


A
LPS / MEDIUM
LPS / LPS
IRF-1
Band Intensity (10^6 pixels)
MAX
(MEDIUM / LPS)
BASAL
(MEDIUM / MEDIUM)
B
IRF-2
Band Intensity (10^5 pixels)
MAX
(MEDIUM / LPS)
BASAL
(MEDIUM / MEDIUM)
C
ICSBP
Band Intensity (10^5 pixels)
MAX
(MEDIUM / LPS)
BASAL
(MEDIUM / MEDIUM)
D
IP-10
Band Intensity (10^6 pixels)
MAX
(MEDIUM / LPS)
BASAL
(MEDIUM / MEDIUM)
Time After LPS Challenge (H)
E
ACTIN

LPS/MEDIUM samples at only 35-50% of maximally LPS-inducible levels. Even after an LPS "challenge," these levels not only fail to be re-induced, but continue to diminish over time in culture. As a control, the effect of endotoxin tolerance on the expression of the IP-10 gene was included. Consistent with previous findings (Henricson *et al.*, 1993), the level of IP-10 gene expression at the time of re-stimulation (LPS/MEDIUM) was very low (about 15% of maximal LPS-induced gene expression), and was not re-induced after subsequent exposure to LPS (LPS/LPS).

Regulation of LPS-inducible gene expression in macrophages derived from IRF-1$^{-/-}$ mice. Because IRF-1 mRNA was readily detectable in unstimulated macrophages (Figure 1) and found to be an immediate-early LPS-inducible gene (Figure 5), we examined the possibility that IRF-1 may be involved in LPS-induced signaling. Macrophages derived from IRF-1$^{-/-}$ mice (IRF-1 "knock-out" mice) and C57BL/6 control mice were stimulated with LPS over time and RNA was analyzed for the expression of several LPS-inducible genes. The results of a representative time course experiment are shown in Figures 7 (Northern blot) and 8 (corresponding PhosphorImager analysis). LPS-inducible TNF-α gene expression was essentially indistinguishable in macrophages derived from the two strains. In contrast, IRF-1$^{-/-}$ macrophages expressed overall lower levels of IL-1β, IRF-2, TNFR-2, and ICSBP in response to LPS than C57BL/6 macrophages. Although maximal expression of LPS-induced IP-10 and IFN-β (data not shown) mRNA were similar in both strains, the levels declined more rapidly in IRF-1$^{-/-}$ macrophages. The pooled geometric means of four similar experiments are depicted in Figure 9, and the following trends were conserved: (1) There was no difference in basal level expression of this panel of genes in macrophages derived from IRF-1$^{-/-}$ and

Figure 7. Representative Northern blot analysis of LPS-induced TNF-α, IL-1β, IP-10, ICSBP, and GAPDH gene expression in macrophages derived from C57BL/6 and IRF-1$^{-/-}$ mice. Macrophages were treated for the indicated periods of time (h) with LPS or with medium only (M). The data shown is derived from a single experiment that is representative of 4 separate experiments.

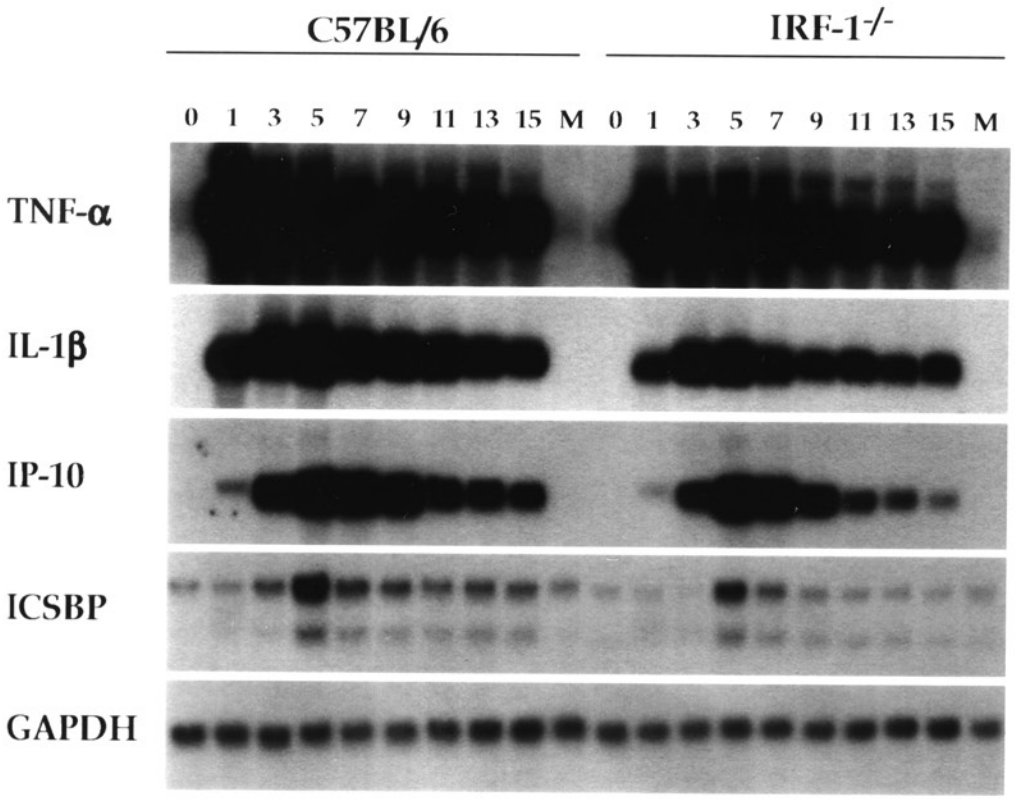
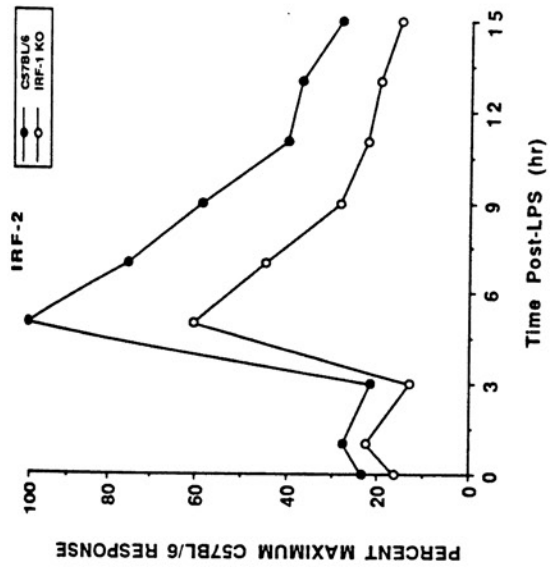
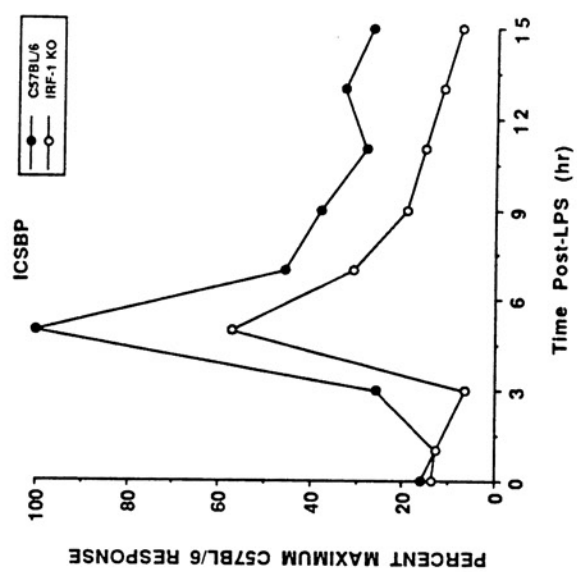
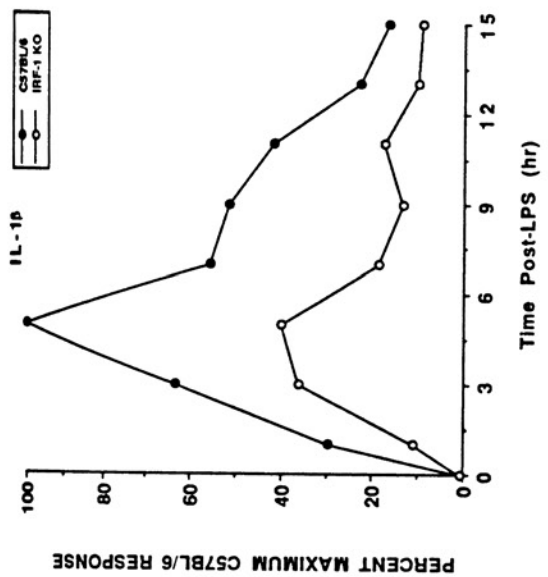
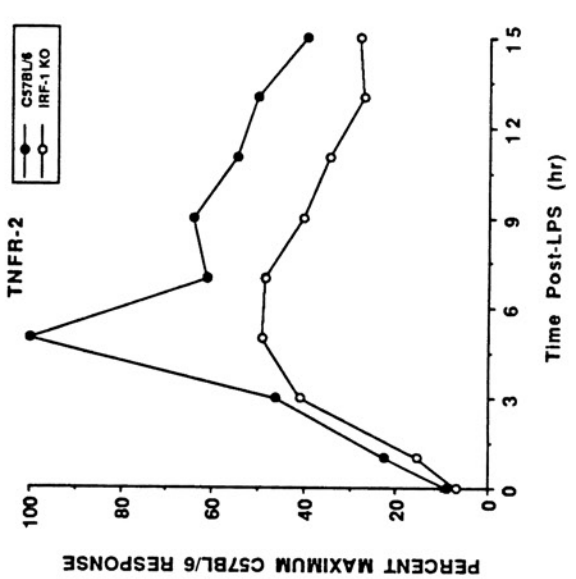

C57BL/6
IRF-1-/-
0 1 3 5 7 9 11 13 15 M 0 1 3 5 7 9 11 13 15 M
TNF-α
IL-1β
IP-10
ICSBP
GAPDH

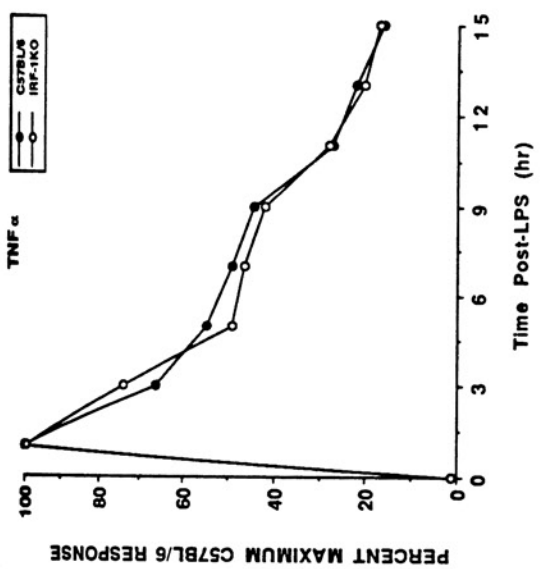
Figure 8. Representative PhosphorImager analysis of LPS-induced TNF-α, IL-1β, IRF-2, IP-10, TNFR-2, and ICSBP gene expression in C57BL/6 and IRF-1$^{-/-}$ macrophages, graphed as percent of maximum LPS-inducible gene expression in control C57BL/6 macrophages (100%). The data is derived from analysis of the Northern blot depicted in Figure 7.


IRF-2
C57BL/6
IRF-1 KO
PERCENT MAXIMUM C57BL/6 RESPONSE
Time Post-LPS (hr)


ICSBP
C57BL/6
IRF-1 KO
PERCENT MAXIMUM C57BL/6 RESPONSE
Time Post-LPS (hr)


IL-1β
C57BL/6
IRF-1 KO
PERCENT MAXIMUM C57BL/6 RESPONSE
Time Post-LPS (hr)


TNFR-2
C57BL/6
IRF-1 KO
PERCENT MAXIMUM C57BL/6 RESPONSE
Time Post-LPS (hr)


TNFα
C57BL/6
IRF-1KO
PERCENT MAXIMUM C57BL/6 RESPONSE
Time Post-LPS (hr)

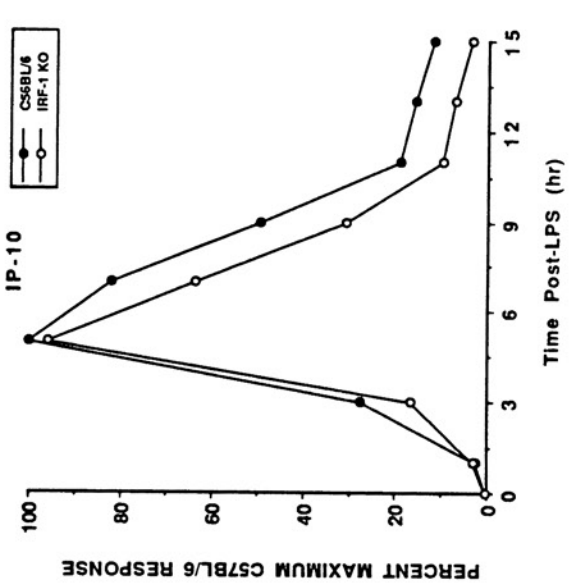
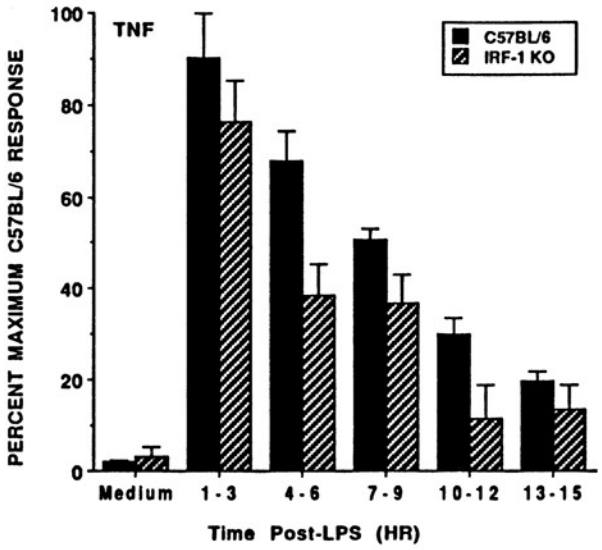
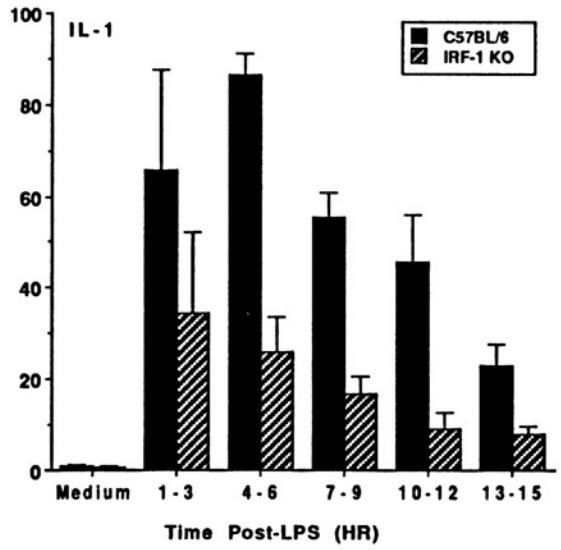
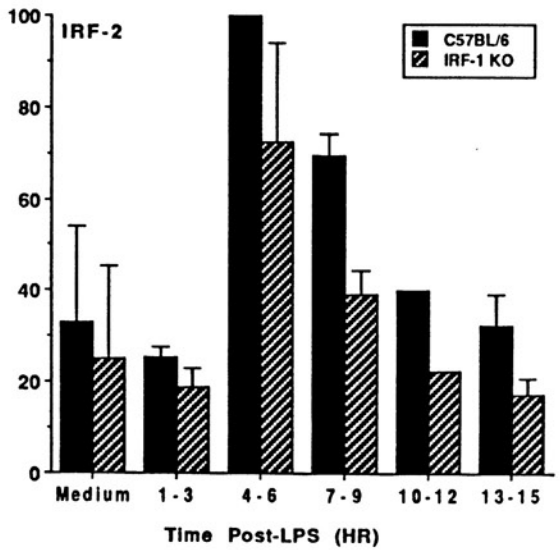
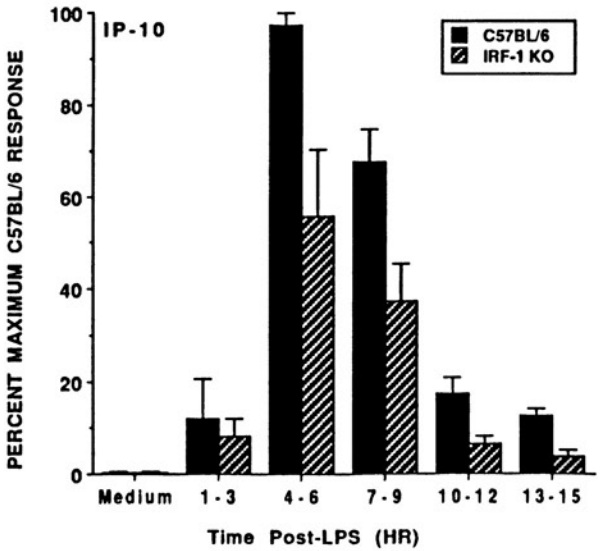

IP-10
C56BL/6
IRF-1 KO
PERCENT MAXIMUM C57BL/6 RESPONSE
Time Post-LPS (hr)

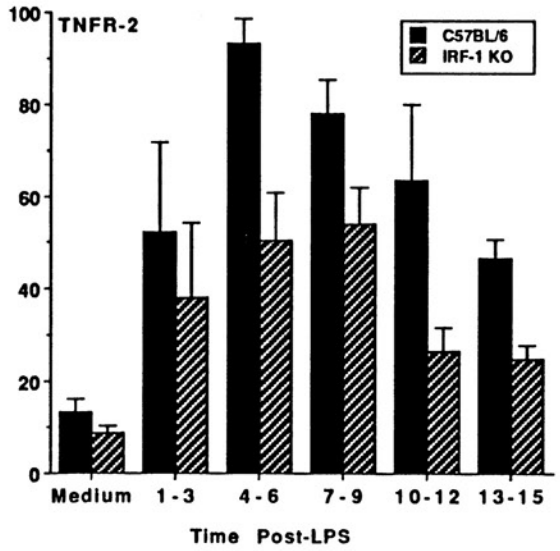
Figure 9. Pooled PhosphorImager analysis of LPS-induced TNF-α, IL-1β, IRF-2, IP-10, TNFR-2, and ICSBP gene expression in IRF-$1^{-/-}$ macrophages graphed as percent of maximum expression in LPS-stimulated control C57BL/6 macrophages (derived from Figure 8). The data is expressed as the pooled geometric mean ± SEM obtained from 4 separate experiments.

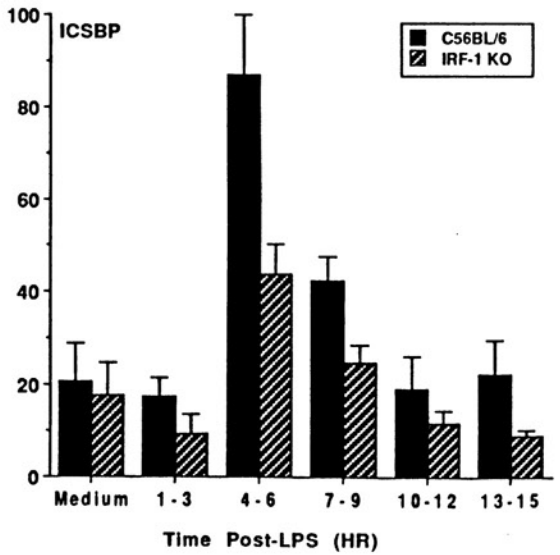

TNF
C57BL/6
IRF-1 KO
PERCENT MAXIMUM C57BL/6 RESPONSE
100
80
60
40
20
0
Medium
1-3
4-6
7-9
10-12
13-15
Time Post-LPS (HR)


IL-1
C57BL/6
IRF-1 KO
100
80
60
40
20
0
Medium
1-3
4-6
7-9
10-12
13-15
Time Post-LPS (HR)


IRF-2
C57BL/6
IRF-1 KO
100
80
60
40
20
0
Medium
1-3
4-6
7-9
10-12
13-15
Time Post-LPS (HR)


IP-10
C57BL/6
IRF-1 KO
PERCENT MAXIMUM C57BL/6 RESPONSE
100
80
60
40
20
0
Medium
1-3
4-6
7-9
10-12
13-15
Time Post-LPS (HR)


TNFR-2
C57BL/6
IRF-1 KO
100
80
60
40
20
0
Medium
1-3
4-6
7-9
10-12
13-15
Time Post-LPS


ICSBP
C56BL/6
IRF-1 KO
100
80
60
40
20
0
Medium
1-3
4-6
7-9
10-12
13-15
Time Post-LPS (HR)

C57BL/6 mice in medium-treated controls or 1 - 3 hr LPS-treated time points, with the exception that IRF-1$^{-/-}$ macrophages expressed lower basal levels of IFN-β mRNA in 5 out of 6 separate comparisons (data not shown); (2) LPS-inducible TNF-α gene expression was the least effected by targeted disruption of the IRF-1 gene; (3) At later time points (i.e., after 4 - 6 h), the expression of IL-1β, IP-10, TNFR-2, and ICSBP genes was lower in IRF-1$^{-/-}$ macrophages, compared to C57BL/6 controls; and (4) IRF-2 and IFN-β (data not shown) gene expression was reduced in IRF-1$^{-/-}$ macrophages after 7-9 hr of LPS stimulation.

Regulation of LPS-inducible gene expression in *in vitro* endotoxin-tolerized macrophages derived from IRF-1$^{-/-}$ mice. To assess a potential role of IRF-1 in the induction or maintenance of endotoxin tolerance, gene expression in macrophages derived from IRF-1$^{-/-}$ or C57BL/6 control mice was analyzed. The results of a representative tolerance experiment are shown in Figures 10 (Northern blot) and 11 (corresponding PhosphorImager analysis). Endotoxin-tolerized IRF-1$^{-/-}$ macrophages re-stimulated with medium alone (M) expressed similar levels of TNF-α, IP-10 and TNFR-2, lower levels of ICSBP and IRF-2, and higher levels of IL-1β mRNA compared to the C57BL/6 controls. Macrophages from both strains exhibited slight re-inducibility of TNF-α and IL-1β genes in response to secondary challenge with LPS (L). The pooled means ± SEMs of four similar experiments, expressed as a percentage of the medium-treated C57BL/6 controls, are shown in Table II and indicate that IRF-1$^{-/-}$ macrophages consistently expressed lower levels of IRF-2, and IFN-β mRNA, slightly lower levels of IP-10, ICSBP and TNFR-2 mRNA, slightly higher levels of TNF-α mRNA, and almost a 6-fold higher level of IL-1β mRNA than endotoxin-tolerized C57BL/6 controls. These findings are

Figure 10. Representative Northern blot analysis of TNF-α, IL-1β, IP-10, ICSBP, and GAPDH gene expression in endotoxin-tolerized macrophages derived from C57BL/6 and IRF-1$^{-/-}$ mice. Macrophages were pre-treated with LPS (endotoxin-tolerized) as described in the Materials and Methods and then re-stimulated with either medium only (M) or LPS (L) for 4 - 6 h. The data shown is derived from a single experiment that is representative of 4 separate experiments.

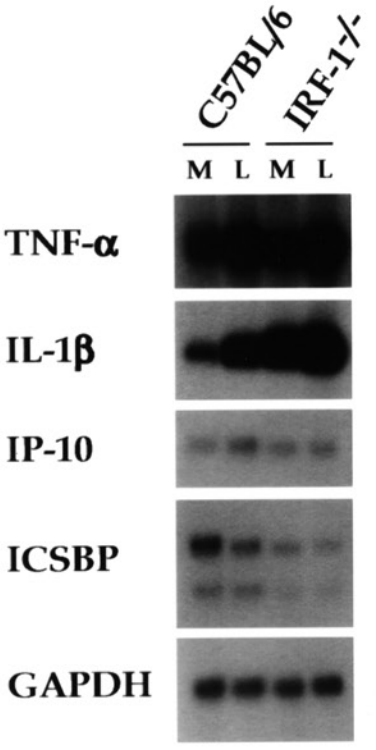

C57BL/6
IRF-1-/-
M L M L
TNF-α
IL-1β
IP-10
ICSBP
GAPDH

Figure 11. Represented PhosphorImager analysis of TNF-α, IL-1β, IRF-2, IP-10, TNFR-2, and ICSBP gene expression in endotoxin-tolerized C57BL/6 control and IRF-1$^{-/-}$ macrophages, graphed as percent of maximum LPS-induced gene expression in non-tolerized C57BL/6 macrophages (derived from Figure 8). The PhosphorImager data is derived from analysis of the same Northern blot experiment depicted in Figure 10.

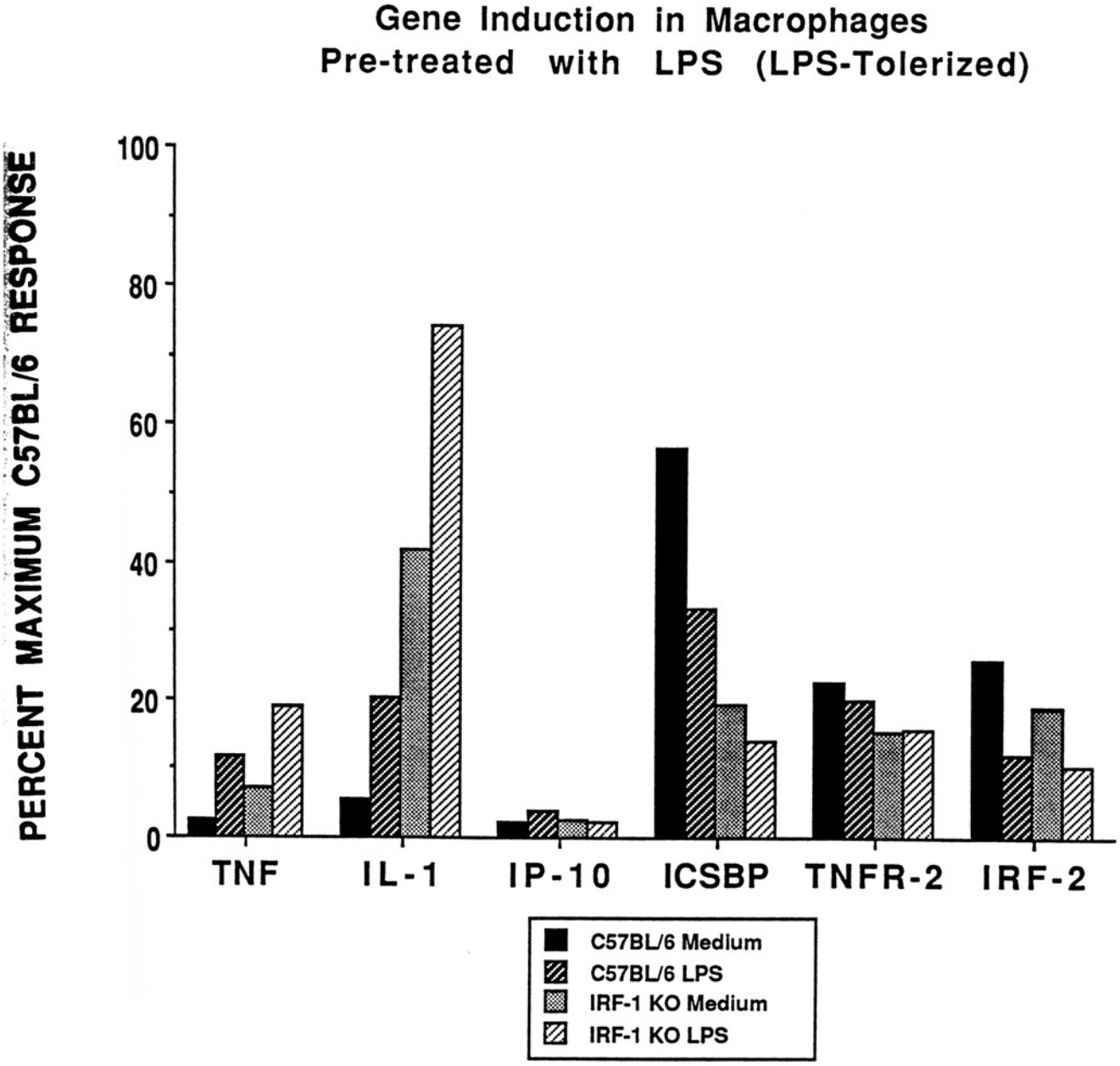

Gene Induction in Macrophages
Pre-treated with LPS (LPS-Tolerized)
PERCENT MAXIMUM C57BL/6 RESPONSE
100
80
60
40
20
0
TNF
IL-1
IP-10
ICSBP
TNFR-2
IRF-2
C57BL/6 Medium
C57BL/6 LPS
IRF-1 KO Medium
IRF-1 KO LPS

Table II: Gene Expression in Endotoxin-Tolerized IRF-1$^{-/-}$ Macrophages[a]

Gene	mean (Range ± SEM)
TNF-α	151.4 (103 - 223)
IL-1β	549.5 (447 - 676)
IP-10	56.9 (35 - 93)
ICSBP	73.3 (44 - 123)
TNFR-2	56.9 (38 - 85)
IRF-2	47.5 (38 - 60)
IFN-β[b]	15.5 (10 - 25)

[a]Macrophages derived from C57BL/6 control or IRF-1$^{-/-}$ mice were tolerized to endotoxin as described in the Materials and Methods and re-stimulated with medium only for 4 - 6 hr. The data are expressed as percent of the endotoxin-tolerized, medium-treated C57BL/6 control macrophages (100%) for each experiment. The values presented were rounded to the nearest whole number and represent the geometric mean ± SEM of 4 separate experiments.

[b]The data were derived from 3 separate experiments and were based on semi-quantitative RT-PCR, rather than Northern analysis.

extended in Figure 12, which shows the data expressed as a percent of the maximum LPS-inducible response observed in non-tolerized C57BL/6 macrophages (denoted by the "O"). Although no gene was appreciably re-induced by LPS, the elevated levels of IL-1β in the medium- or LPS-restimulated IRF-1$^{-/-}$ macrophages were very close to (medium) or higher than (LPS) the maximum LPS-induced levels in non-tolerized IRF-1$^{-/-}$ macrophages (denoted by the "X").

Regulation of LPS-inducible gene expression in macrophages derived from IRF-2$^{-/-}$ mice. To assess a potential role for IRF-2 in regulating LPS-inducible gene expression, macrophages derived from IRF-2$^{-/-}$ (IRF-2 "knock-out") and C57BL/6 control mice were stimulated with LPS and the resulting patterns of gene expression were compared. Gene expression profiles from a representative time course experiment are depicted in Figures 13 (Northern blot) and 14 (corresponding PhosphorImager analysis). Overall, the relative levels of each gene were lower in the IRF-2$^{-/-}$ macrophages; however, IL-1β, IP-10, TNFR-2, and ICSBP genes were affected most by targeted disruption of the IRF-2 gene. The pooled geometric means of three separate experiments are shown in Fig. 15, and the following trends were conserved: (1) There was no difference in basal level expression of any gene examined, with the possible exception of IFN-β (in 4 of 4 comparisons, IRF-2$^{-/-}$ macrophages contained slightly higher basal levels of IFN-β, data not shown); (2) IRF-1 and IFN-β (data not shown) gene expression in LPS-stimulated IRF-2$^{-/-}$ macrophages was very similar to that observed in the C57BL/6 controls; (3) TNF-α, IL-1β, IP-10 and ICSBP gene expression was lower after 4 h of LPS stimulation in IRF-2$^{-/-}$ macrophages, compared to the C57BL/6 controls, while the expression of TNFR-2 was lower in the IRF-2$^{-/-}$ macrophages essentially throughout the time course.

Figure 12. Pooled PhosphorImager analysis of TNF-α, IL-1β, IRF-2, IP-10, TNFR-2 and ICSBP gene expression in endotoxin-tolerized C57BL/6 control and IRF-1$^{-/-}$ macrophages, graphed as percent of maximum LPS-induced gene expression in non-tolerized C57BL/6 macrophages (derived from Figure 8). The data is expressed as the pooled geometric mean ± SEM obtained from 4 separate experiments. "O" represents the mean maximum mRNA level of LPS-stimulated (non-tolerized) C57BL/6 macrophages. "X" represents the mean maximum mRNA level of LPS-stimulated (non-tolerized) IRF-1$^{-/-}$ macrophages.

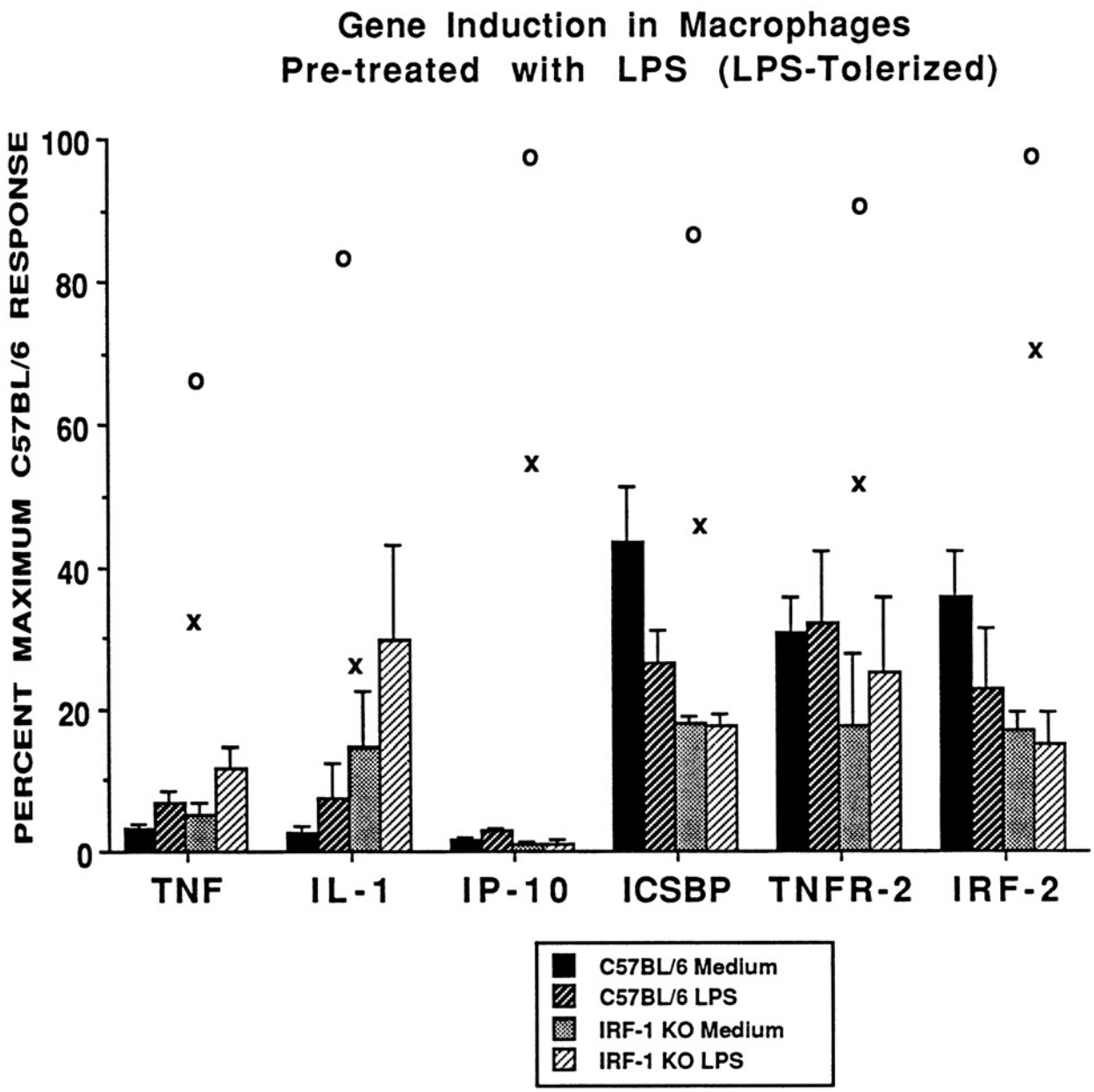

Gene Induction in Macrophages
Pre-treated with LPS (LPS-Tolerized)
PERCENT MAXIMUM C57BL/6 RESPONSE
100
80
60
40
20
0
TNF
IL-1
IP-10
ICSBP
TNFR-2
IRF-2
C57BL/6 Medium
C57BL/6 LPS
IRF-1 KO Medium
IRF-1 KO LPS

Figure 13. Representative Northern blot analysis of LPS-induced TNF-α, IL-1β, IP-10, TNFR-2, IRF-1, ICSBP, and GAPDH gene expression in macrophages derived from C57BL/6 and IRF-2$^{-/-}$ mice. Macrophages were treated over time with LPS as described in the Materials and Methods. The data shown is derived from a single experiment that is representative of 3 separate experiments.

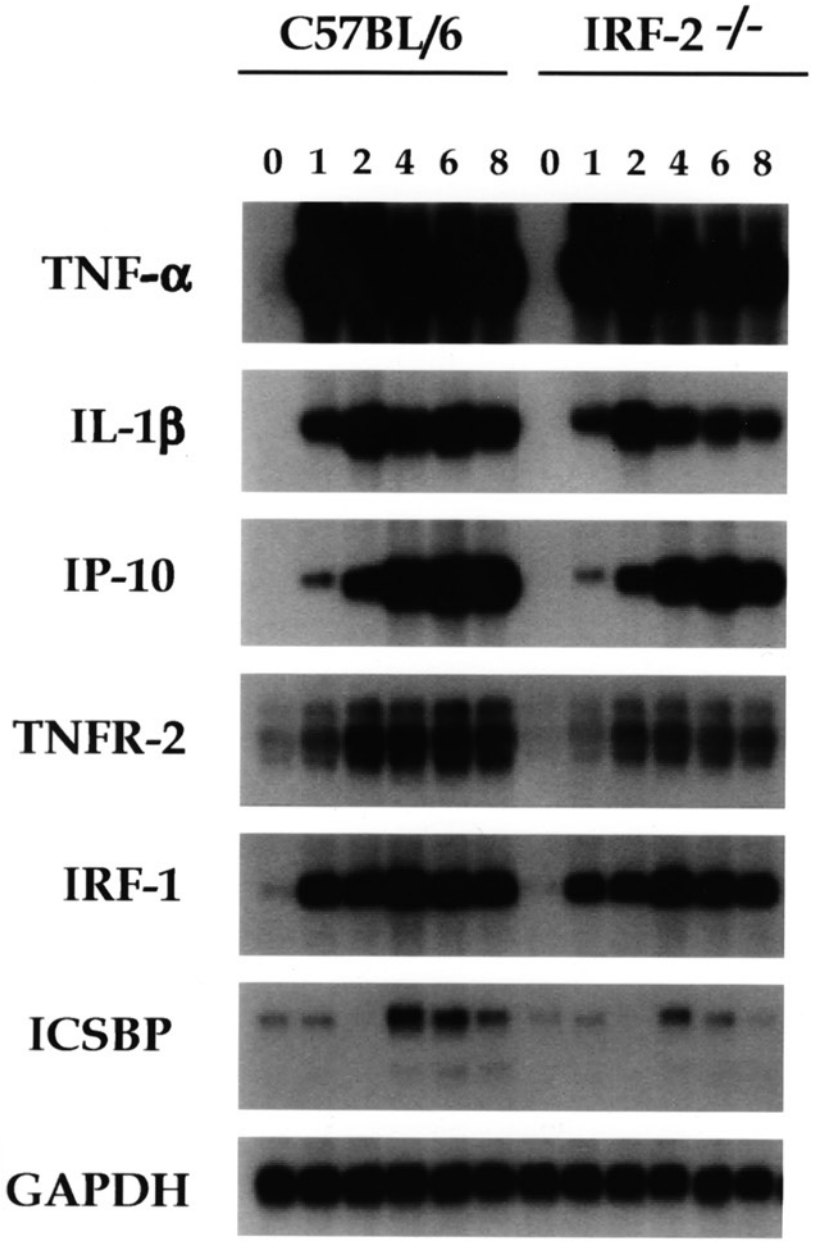
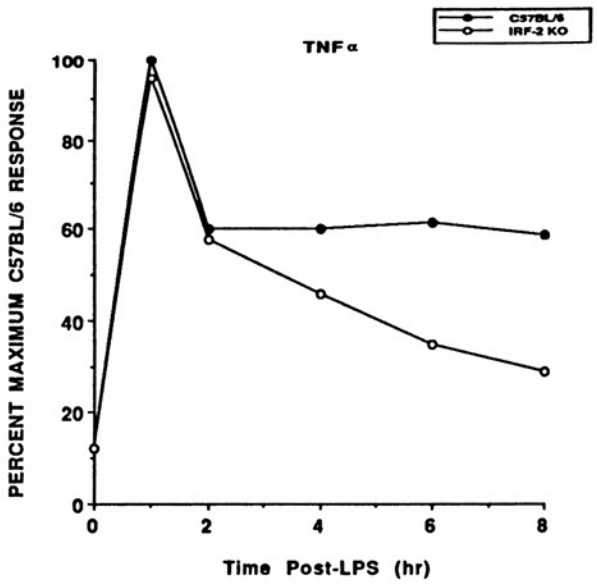
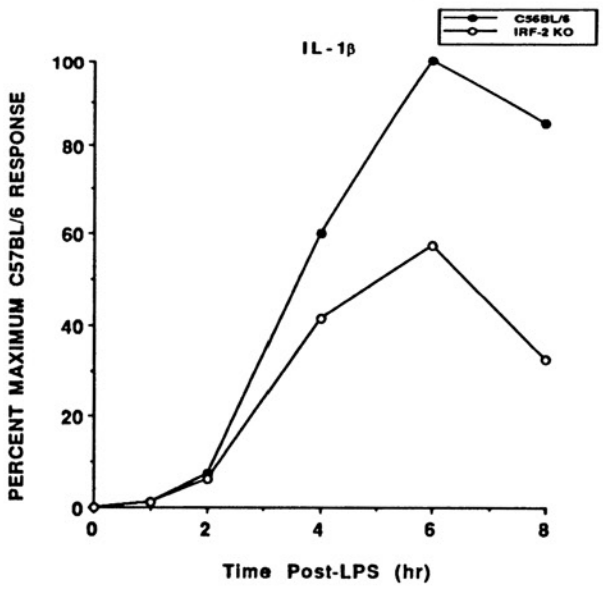
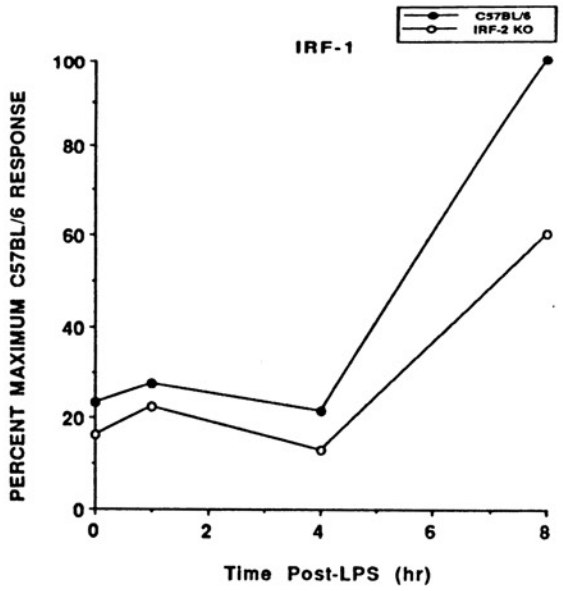
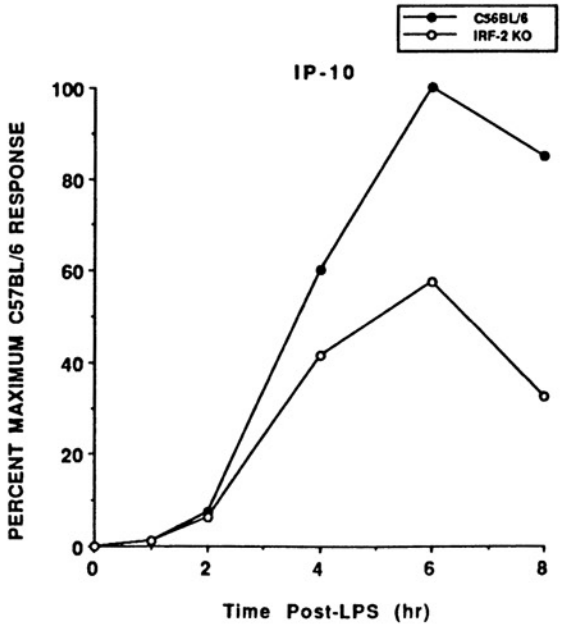

C57BL/6
IRF-2 -/-
0 1 2 4 6 8 0 1 2 4 6 8
TNF-α
IL-1β
IP-10
TNFR-2
IRF-1
ICSBP
GAPDH

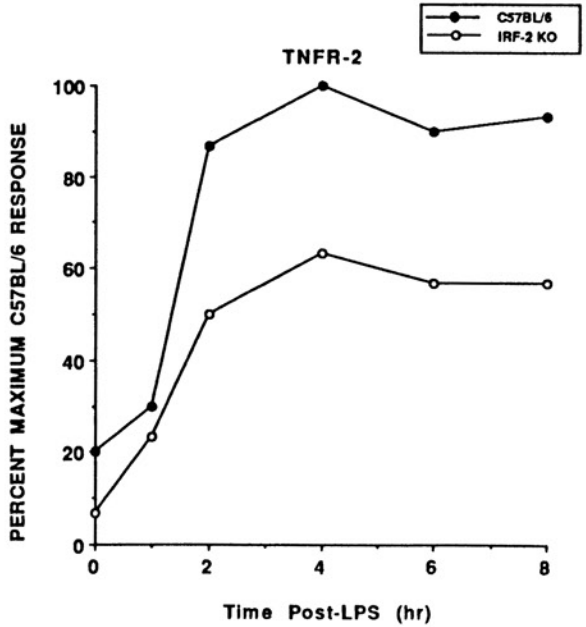
Figure 14. Representative PhosphorImager analysis of LPS-induced TNF-α, IL-1β, IRF-1, IP-10, TNFR-2, and ICSBP gene expression in IRF-2$^{-/-}$ macrophages graphed as percent of maximum LPS-inducible gene expression in control C57BL/6 macrophages (100%). The data is derived from the analysis of the Northern blot depicted in Figure 13.


TNFα
C57BL/6
IRF-2 KO
PERCENT MAXIMUM C57BL/6 RESPONSE
Time Post-LPS (hr)


IL-1β
C56BL/6
IRF-2 KO
PERCENT MAXIMUM C57BL/6 RESPONSE
Time Post-LPS (hr)


IRF-1
C57BL/6
IRF-2 KO
PERCENT MAXIMUM C57BL/6 RESPONSE
Time Post-LPS (hr)


IP-10
C56BL/6
IRF-2 KO
PERCENT MAXIMUM C57BL/6 RESPONSE
Time Post-LPS (hr)


TNFR-2
C57BL/6
IRF-2 KO
PERCENT MAXIMUM C57BL/6 RESPONSE
Time Post-LPS (hr)

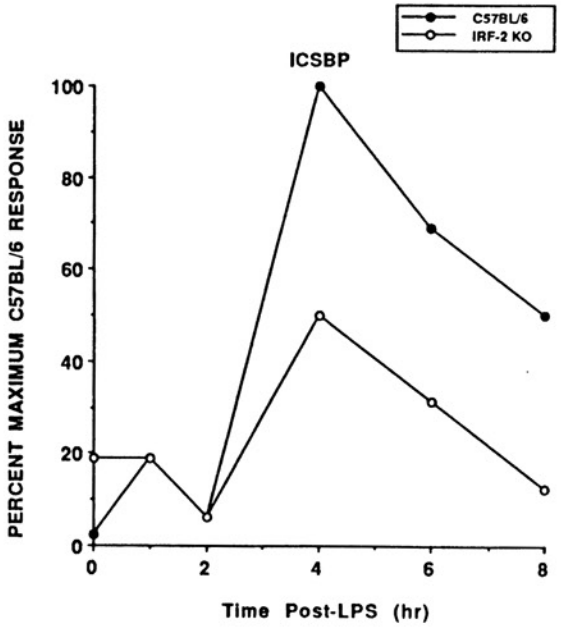
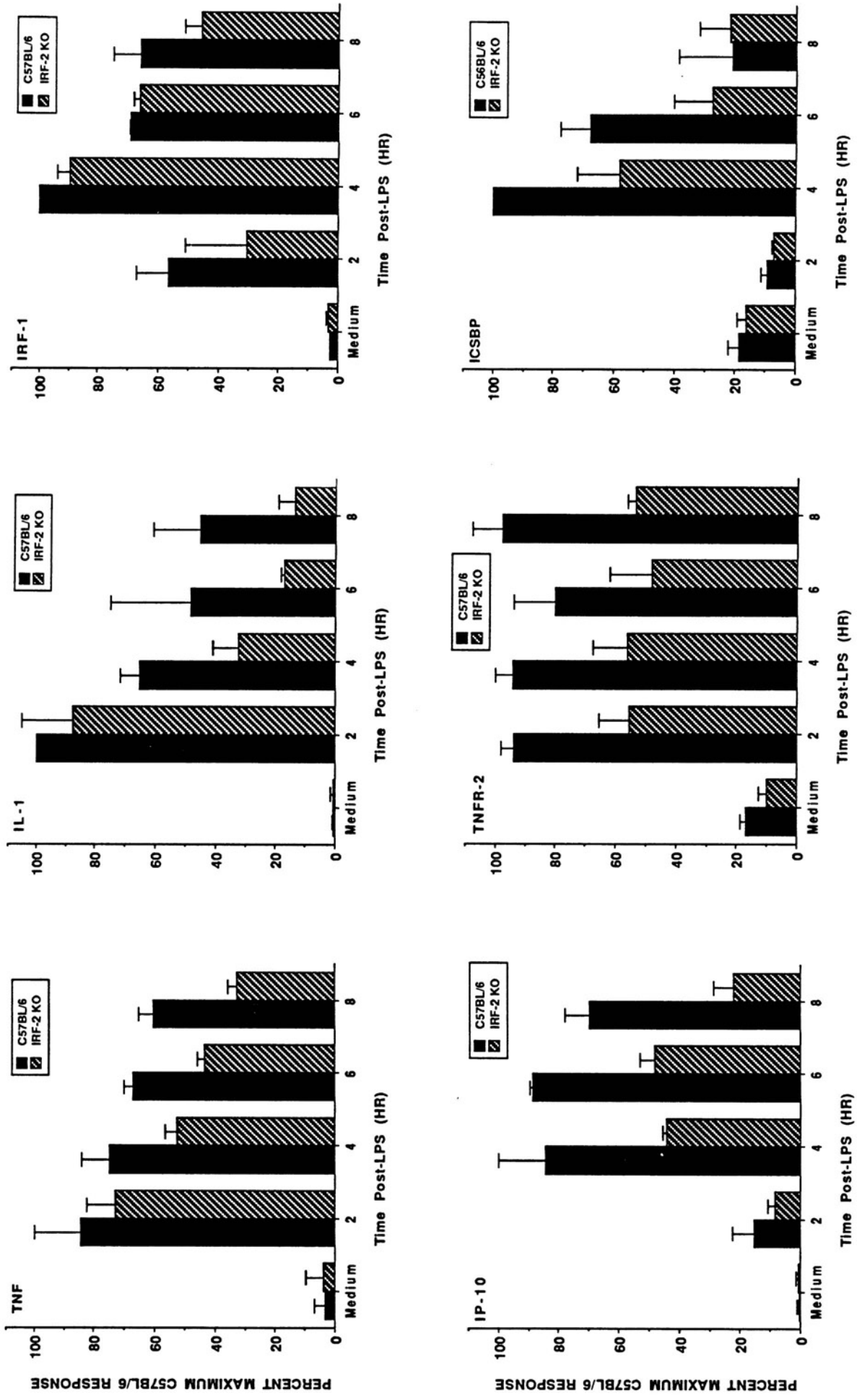

ICSBP
C57BL/6
IRF-2 KO
PERCENT MAXIMUM C57BL/6 RESPONSE
Time Post-LPS (hr)

Figure 15. Pooled PhosphorImager analysis of LPS-induced TNF-α, IL-1β, IRF-1, IP-10, TNFR-2, and ICSBP gene expression in IRF-$2^{-/-}$ macrophages graphed as percent of maximum expression in LPS-stimulated control C57BL/6 macrophages (derived from Figure 14). The data is expressed as the pooled geometric mean ± SEM obtained from 3 separate experiments.


TNF
IL-1
IRF-1
TNFR-2
ICSBP
IP-10
C57BL/6
IRF-2 KO
C56BL/6
IRF-2 KO
PERCENT MAXIMUM C57BL/6 RESPONSE
Time Post-LPS (HR)
Medium
0
20
40
60
80
100
2
4
6
8

Regulation of LPS-inducible gene expression in *in vitro* endotoxin-tolerized macrophages derived from IRF-2$^{-/-}$ mice. To assess a potential role of IRF-2 in induction or maintenance of endotoxin tolerance, gene expression in macrophages derived from IRF-2$^{-/-}$ or C57BL/6 control mice was analyzed. The results of a representative *in vitro* tolerance experiment are shown in Figures 16 (Northern blot) and 17 (corresponding PhosphorImager analysis). Endotoxin-tolerized IRF-2$^{-/-}$ macrophages re-stimulated with medium only (M) expressed slightly lower levels of IRF-1, TNFR-2, and ICSBP mRNA, substantially lower levels of IP-10 mRNA, slightly higher levels of TNF-α, and substantially higher levels of IL-1β mRNA compared to the endotoxin-tolerized the C57BL/6 controls. Although both TNF-α and IL-1β were slightly increased upon secondary challenge with LPS, no gene was differentially re-inducible by LPS in endotoxin-tolerized macrophages derived from the two strains. The pooled means of three separate experiments, expressed as a percentage of the medium-treated C57BL/6 controls are shown in Table III and indicate that IRF-2$^{-/-}$ macrophages consistently expressed higher levels of IL-1β, similar levels of TNF-α, and somewhat lower levels of IRF-1, ICSBP, and TNFR-2 mRNA, than the C57BL/6 controls. IP-10 gene expression was very low in tolerized IRF-2$^{-/-}$ macrophages, whereas the expression of IFN-β was de-repressed in comparison to endotoxin-tolerized C57BL/6 controls. These findings are extended in Figure 18, which shows the data expressed as a percent of the maximum LPS-inducible response observed in the non-tolerized C57BL/6 macrophages. The expression of each gene is repressed in comparison to the maximally induced levels in non-tolerized macrophages from both strains, and no gene was appreciably re-induced by LPS.

Figure 16. Representative Northern blot analysis of LPS-induced TNF-α, IL-1β, IP-10, TNFR-2, IRF-1, ICSBP, and GAPDH gene expression in endotoxin-tolerized macrophages derived from C57BL/6 and IRF-2$^{-/-}$ mice. Macrophages were pre-treated with LPS (endotoxin-tolerized) as described in the Materials and Methods and then re-stimulated with either medium only (M) or LPS (L) for 4 - 6 h. The data shown is derived from a single experiment that is representative of 3 separate experiments.

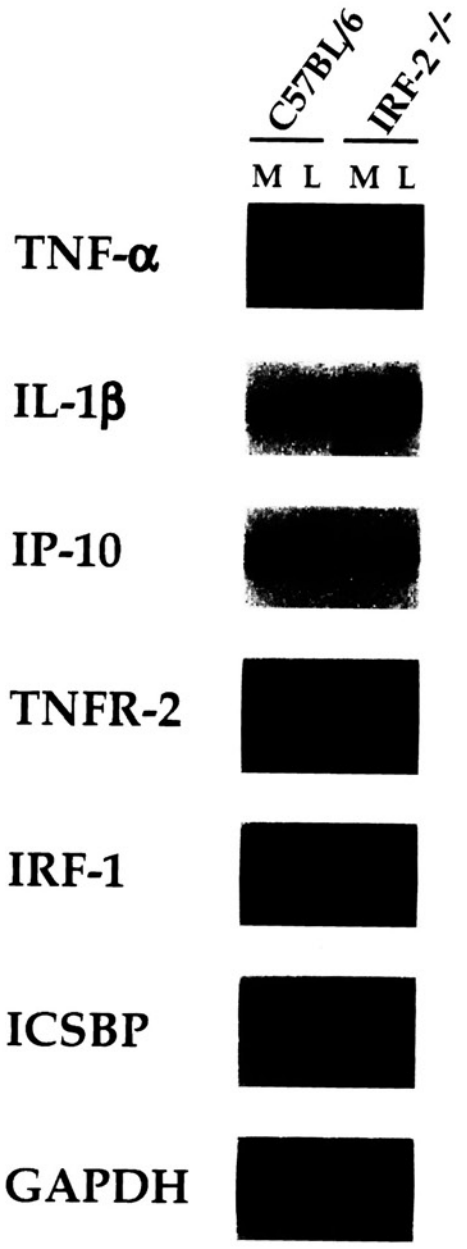

C57BL/6
IRF-2 -/-
M L M L
TNF-α
IL-1β
IP-10
TNFR-2
IRF-1
ICSBP
GAPDH

Figure 17. Representative PhosphorImager analysis of LPS-induced TNF-α, IL-1β, IP-10, TNFR-2, IRF-1, and ICSBP gene expression in endotoxin-tolerized IRF-2$^{-/-}$ macrophages graphed as percent of maximum LPS-induced gene expression in non-tolerized C57BL/6 macrophages (derived from Figure 14). The PhosphorImager data is derived from analysis of the same Northern blot experiment depicted in Figure 16.

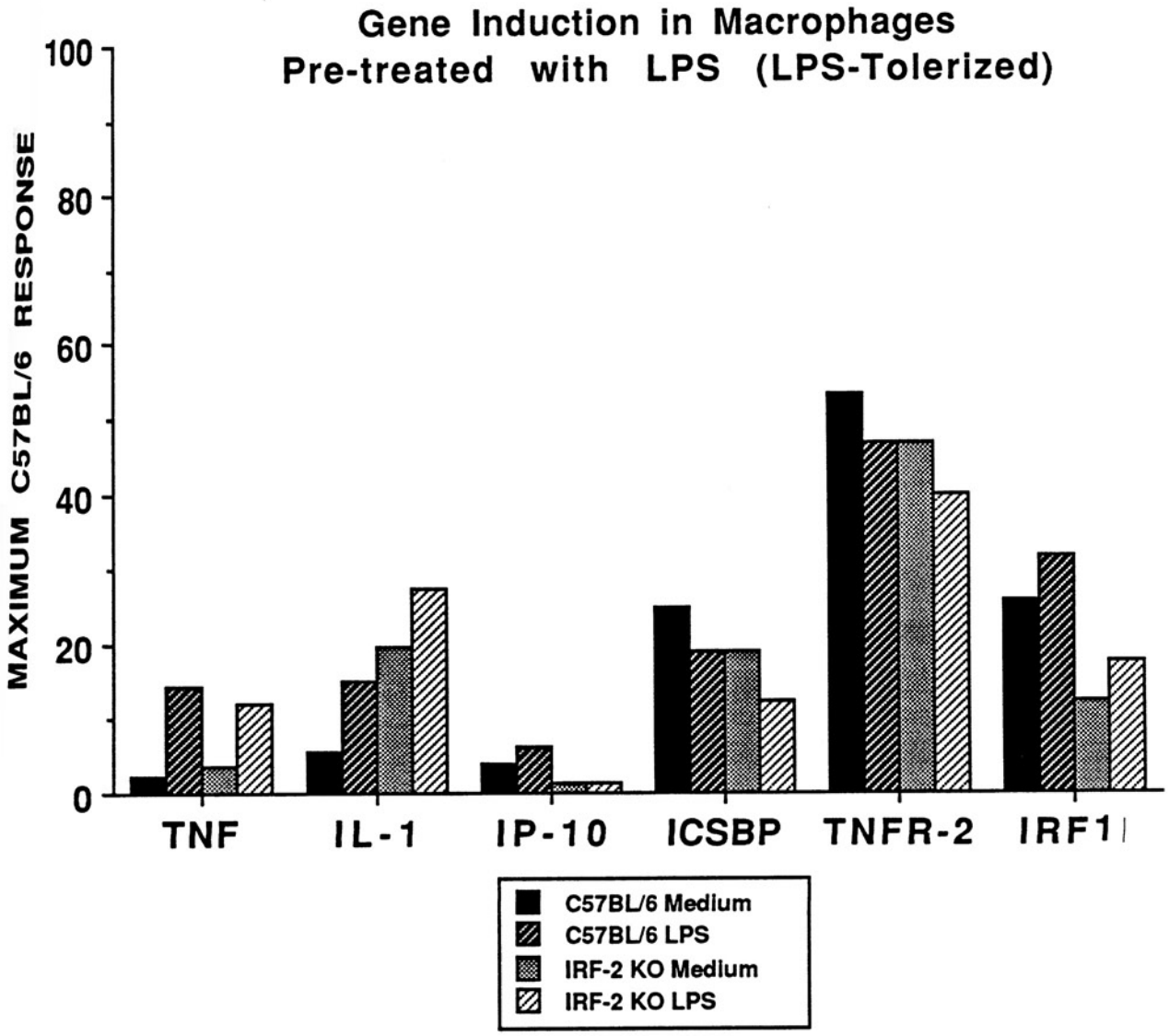

Gene Induction in Macrophages
Pre-treated with LPS (LPS-Tolerized)
MAXIMUM C57BL/6 RESPONSE
100
80
60
40
20
0
TNF
IL-1
IP-10
ICSBP
TNFR-2
IRF1
C57BL/6 Medium
C57BL/6 LPS
IRF-2 KO Medium
IRF-2 KO LPS

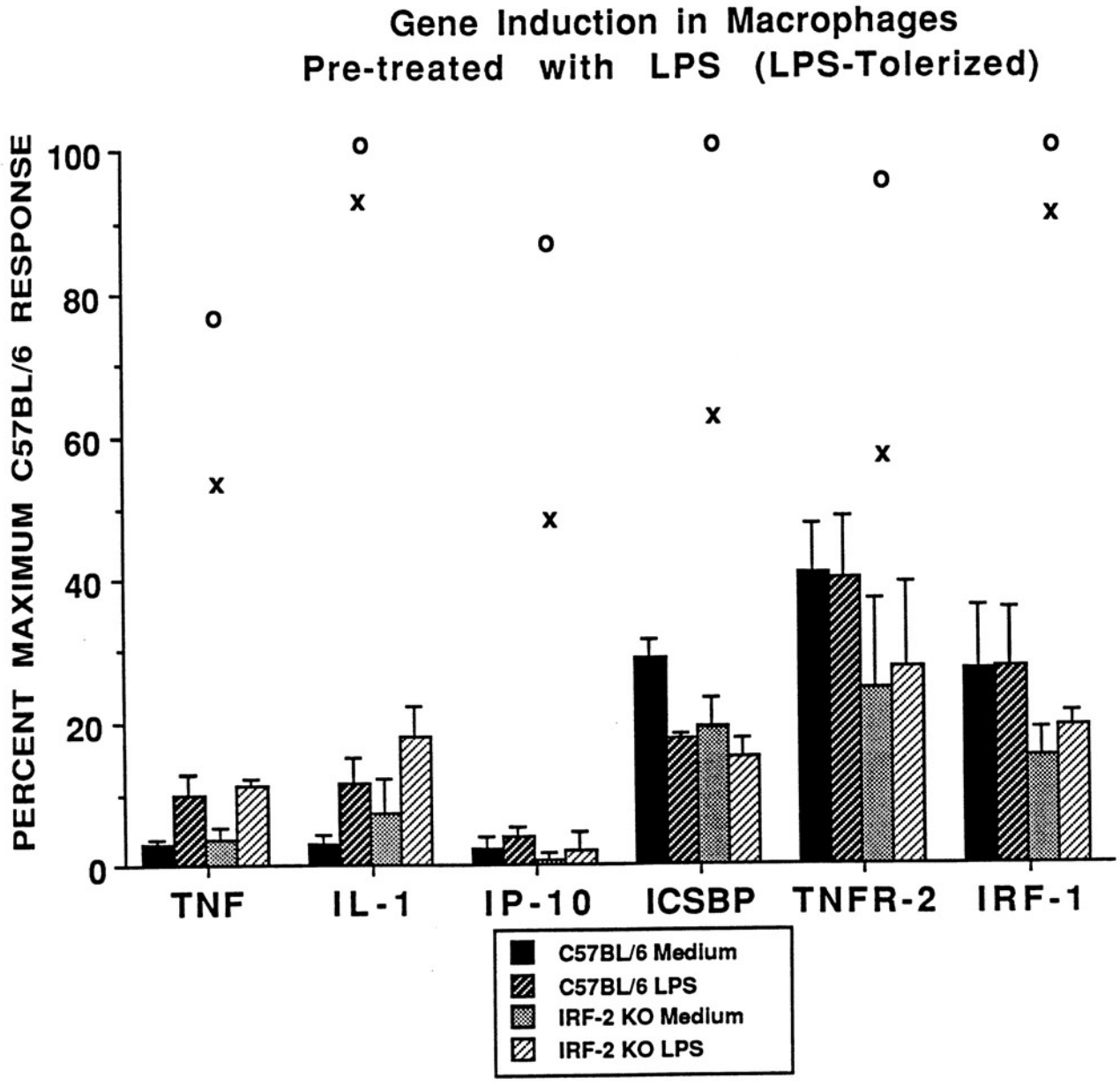
Table III: Gene Expression in Endotoxin-Tolerized IRF-2$^{-/-}$ Macrophages[a]

Gene	mean (Range ± SEM)
TNF-α	123.9 (101 - 151)
IL-1β	245.5 (176 - 343)
IP-10	30.4 (24 - 39)
ICSBP	64.1 (57 - 72)
TNFR-2	58.5 (57 - 60)
IRF-1	55.3 (50 - 61)
IFN-β[b]	788.9 (776 - 802)

[a]Macrophages derived from C57BL/6 control or IRF-2$^{-/-}$ mice were tolerized to endotoxin as described in the Materials and Methods and re-stimulated with medium only for 4 - 6 hr. The data are expressed as percent of the endotoxin-tolerized, medium-treated C57BL/6 control macrophages (100%) for each experiment. The values presented were rounded to the nearest whole number and represent the geometric mean ± SEM of 3 separate experiments.

[b]The data is expressed as mean ± standard deviation and were derived from 2 separate experiments and were based on semi-quantitative RT-PCR, rather than Northern analysis.

Figure 18. Pooled PhosphorImager analysis of LPS-induced TNF-α, IL-1β, IP-10, ICSBP, TNFR-2 and IRF-1 gene expression in endotoxin-tolerized IRF-$2^{-/-}$ macrophages graphed as percent maximum expression in control C57BL/6 macrophages (derived from Figure 14). The data is expressed as the pooled geometric mean ± SEM obtained from 3 separate experiments.


Gene Induction in Macrophages
Pre-treated with LPS (LPS-Tolerized)
PERCENT MAXIMUM C57BL/6 RESPONSE
100
80
60
40
20
0
TNF
IL-1
IP-10
ICSBP
TNFR-2
IRF-1
C57BL/6 Medium
C57BL/6 LPS
IRF-2 KO Medium
IRF-2 KO LPS

ANALYSIS OF THE POTENTIAL ROLE OF SERINE/THREONINE PHOSPHATASES IN LPS-INDUCED SIGNALING LEADING TO GENE EXPRESSION

As presented in the Introduction, LPS-stimulated macrophages produce inflammatory mediators that are largely responsible for the pathophysiology associated with septic shock. Macrophages respond to LPS by transiently activating a variety of intracellular pathways. These pathways are characterized, in part, by rapid protein phosphorylation and dephosphorylation on serine, threonine, and tyrosine residues. If these events are critical for the cellular response to LPS, the kinases and/or phosphatases involved may be vulnerable targets for pharmacologic intervention in sepsis. In support of this hypothesis are the recent studies (described above) that demonstrated that tyrosine kinase inhibitors block LPS-induced tyrosine phosphorylation of MAP kinases, antagonize TNF-α and IL-1β production, and protect mice from the lethal effects of endotoxin. The experiments described below were designed to investigate a possible role for ser/thr phosphatases in LPS signaling leading to gene expression. We therefore evaluated the effects of calyculin A, and okadaic acid, two potent ser/thr phosphatase inhibitors, on LPS signaling leading to gene expression in murine macrophages. Calyculin A and okadaic acid are potent, and distinguishable inhibitors of protein phosphatases (PP) 1 and PP2A, two of the most abundant ser/thr phosphatases in the cell (Cohen, 1989; Suganuma *et al.*, 1990; Cohen *et al.*, 1990).

Effect of the serine/threonine phosphatase inhibitor, calyculin A, on LPS-induced gene expression and TP. To assess the role of ser/thr phosphatases in LPS-signal transduction, the dose- and time-dependent inhibition of cellular phosphatase

activity by calyculin A was first evaluated. Peritoneal macrophages were cultured over time with various doses of calyculin A or 100 ng/ml LPS, and cell lysates were extracted and analyzed for phosphatase activity as described in the Materials and Methods. Based on preliminary dose response analyses (Table IV), 25 nM calyculin A was used in all subsequent experiments. This dose had no adverse effect on macrophage viability, as assessed by trypan blue exclusion or adherence, as determined microscopically (data not shown). Figure 19 shows a comparison of phosphatase activity in LPS- and calyculin A-treated macrophages over time. The data are expressed as a percent of medium-treated controls and the phosphatase activity of the medium-treated control group varied <5% over the 12 h examined. The results indicate that LPS did not induce a major fluctuation in total macrophage ser/thr phosphatase activity (<9% variation in three separate experiments). In contrast, calyculin A caused a rapid and sustained decrease in phosphatase activity, which approached maximal inhibition (83 - 94% in three separate experiments) between 30 min and 1 h.

As presented in the Introduction, LPS stimulation of macrophages results in the expression of many genes, among which are the immediate-early genes TNF-α, IL-1β, IFN-β, IP-10, IRF-1, and TNFR-2 (Tannenbaum *et al.*, 1988; Manthey *et al.*, 1992; Barber *et al.*, 1994). Therefore, we sought to evaluate the effect of calyculin A on the expression of these genes as an indication of the integrity of the LPS signal transduction pathway(s). In doing so, the times at which the calyculin A was added to the cultures relative to the LPS were varied in the hope of distinguishing multiple phosphatase activity requirements. Regardless of the time that calyculin A was added relative to LPS, the RNA was harvested 4.5 h after LPS stimulation. This time point was chosen since it allowed for near-optimal detection of each of the immediate-early genes examined (Tannenbaum *et al.*, 1988; Manthey *et al.*, 1992;

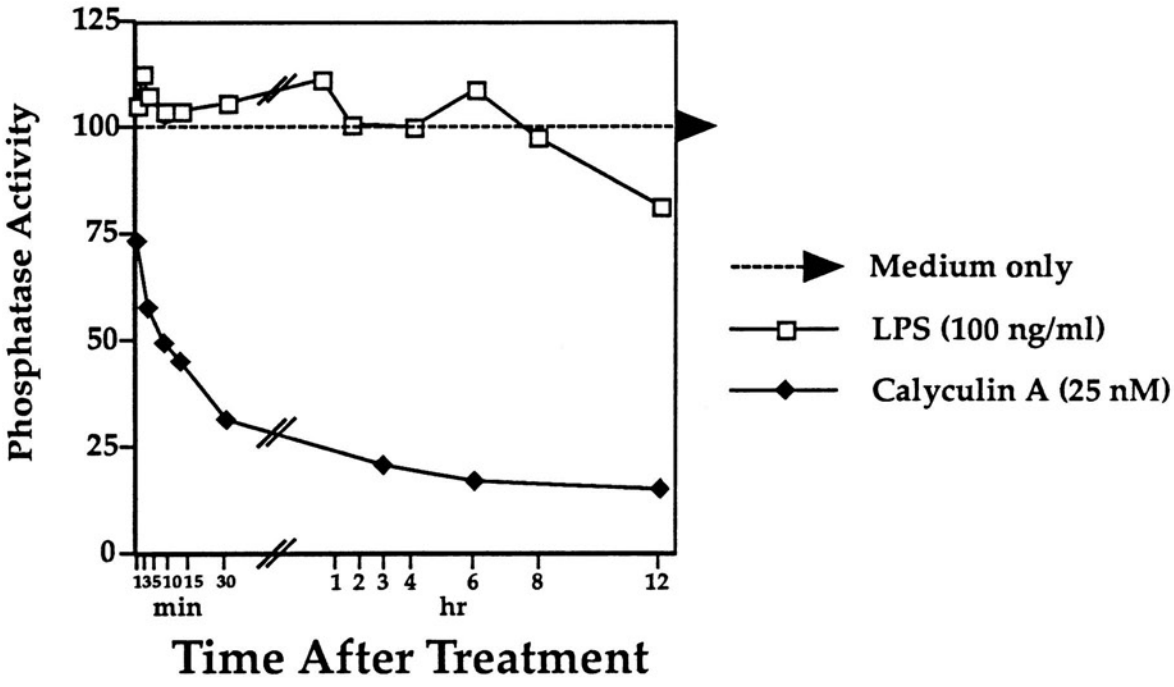
Table IV: Sensitivity of Macrophage Phosphatase Activity to Calyculin A[a]

Concentration of Calyculin A	% Phosphatase Activity[b]
0.25 nM	99 ± 4.8
2.5 nM	98 ± 9.6
25 nM	29 ± 1.1

[a]Macrophages were treated as described in the Materials and Methods with the indicated concentrations of calyculin A for 30 - 60 min.

[b]Data represent the arithmetic mean ± SEM of 3 separate experiments. The data are expressed as percent of the medium-treated controls for each experiment.

Figure 19. Phosphatase activity in macrophages treated with LPS or calyculin A. Whole cell lysates of peritoneal exudate macrophages from C3H/OuJ mice were prepared and assayed for phosphatase activity as described in Materials and Methods. Phosphatase activity is expressed as a percent of medium-treated controls and the phosphatase activity of the control group varied <5% over time. The data shown is derived from a single experiment that is representative of 3 separate experiments.


125
100
75
50
25
0
Phosphatase Activity
1 3 5 10 15 30
min
1 2 3 4 6 8 12
hr
Time After Treatment
Medium only
LPS (100 ng/ml)
Calyculin A (25 nM)

Barber *et al.*, 1993). The results are shown in Figures 20 and 21. Consistent with a previous report (Sung *et al.*, 1992), calyculin A alone induced low, but detectable, expression of TNF-α mRNA. Calyculin A also induced low levels of IP-10, IRF-1, and IFN-β mRNA; however, LPS alone induced high levels of expression of each gene. Pre-treatment of macrophages with calyculin A for one hour prior to LPS stimulation (-1 h) resulted in a gene expression pattern no different than that achieved by calyculin A alone. Simultaneous addition of LPS and calyculin A (0 h) did not inhibit LPS-induced TNF-α, IL-1β, or IFN-β gene expression, but substantially lowered mRNA levels of IP-10, IRF-1, and TNFR-2. Addition of calyculin A 1.5 h after LPS stimulation again resulted in markedly reduced levels of IP-10, IRF-1, and TNFR-2 mRNA, and the effect of calyculin A on the expression of these three genes was essentially the same whether added simultaneously with or 1.5 h after LPS. In contrast, calyculin A, added 1.5 h post-LPS stimulation, appeared to increase levels of TNF-α and IFN-β mRNA (above those observed with LPS only), but had no effect on LPS-induced levels of IL-1β mRNA. Statistical analysis of the cumulative data from 3 separate experiments is shown in Table V and supports the hypothesis that LPS-induced gene expression is dependent on calyculin A-sensitive phosphatases. To determine if calyculin A affects gene expression post-transcriptionally, TNF and IFN bioassays were performed on macrophage culture supernatants collected at the same time as described for the mRNA analyses. As shown in Table VI, supernatants from macrophages pre-treated with calyculin A 1 h prior to the addition of LPS exhibited bioactivities for TNF and IFN that were suppressed to near control levels (i.e., medium only, DMSO only, and calyculin A only). In contrast, supernatants from macrophages treated with calyculin A 1.5 h after the addition of LPS, showed TNF and IFN bioactivities that were not measurably different (i.e., greater than two-fold) from macrophages treated with LPS alone. The synergy observed between calyculin A and LPS for TNF and IFN at the mRNA level in the post-treatment samples

Figure 20. TNF-α, IL-1β, IP-10, IRF-1, TNFR-2, IFN-β, and GAPDH gene expression in C3H/OuJ macrophages treated as indicated with 25 nM calyculin A, and/or 100 ng/ml LPS. In the samples where calyculin A was added before (-1 h), simultaneously with (0 h), or after (+1.5 h) LPS, RNA was harvested 4.5 h after the LPS was added. Statistical analysis of three separate experiments is provided in Table III.

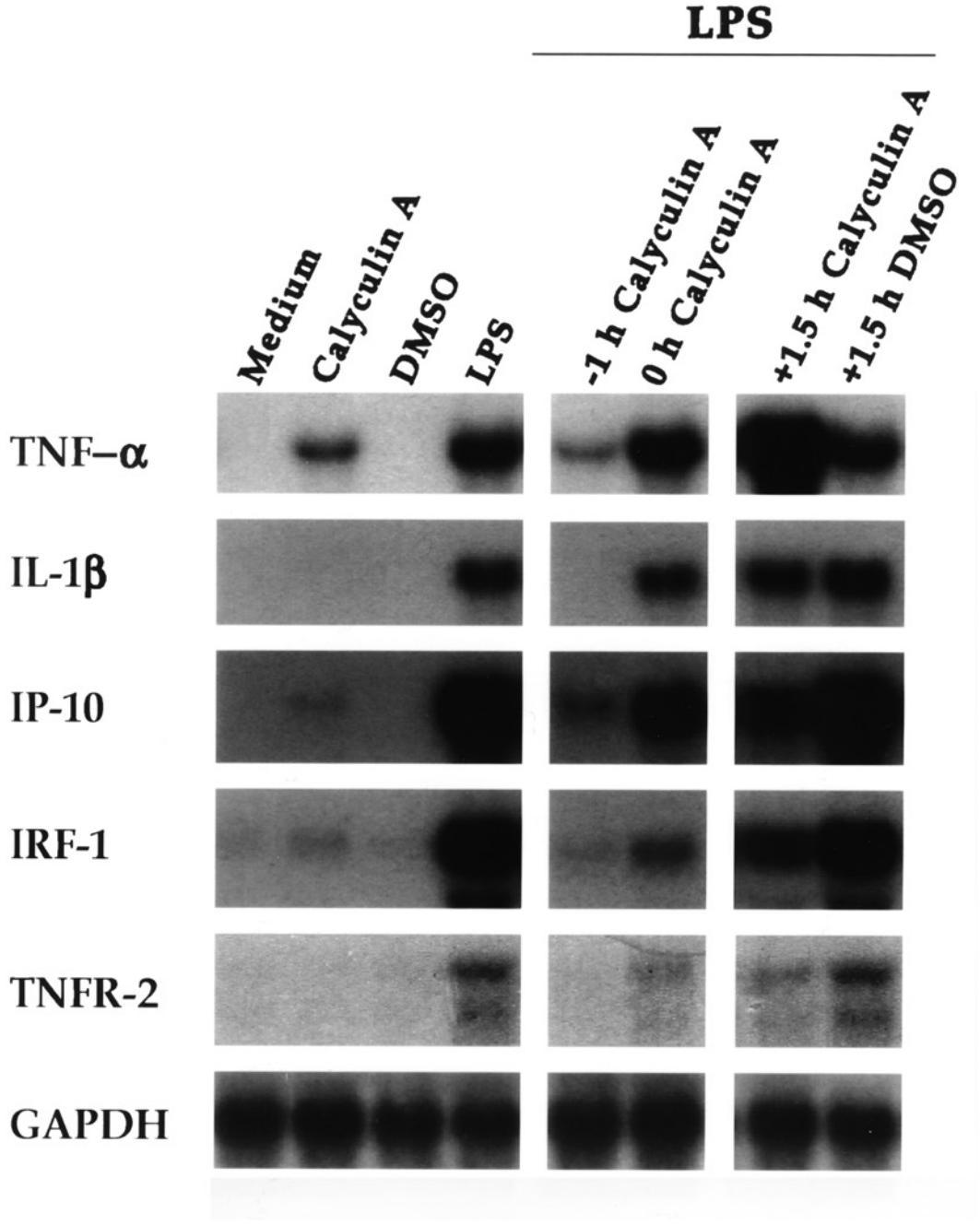

LPS
Medium
Calyculin A
DMSO
LPS
-1 h Calyculin A
0 h Calyculin A
+1.5 h Calyculin A
+1.5 h DMSO
TNF–α
IL-1β
IP-10
IRF-1
TNFR-2
GAPDH

Figure 21. RT-PCR analysis of IFN-β (30 cycles) and GAPDH (20 cycles) gene expression in C3H/OuJ macrophages treated as indicated with 25 nM calyculin A, and/or 100 ng/ml LPS.

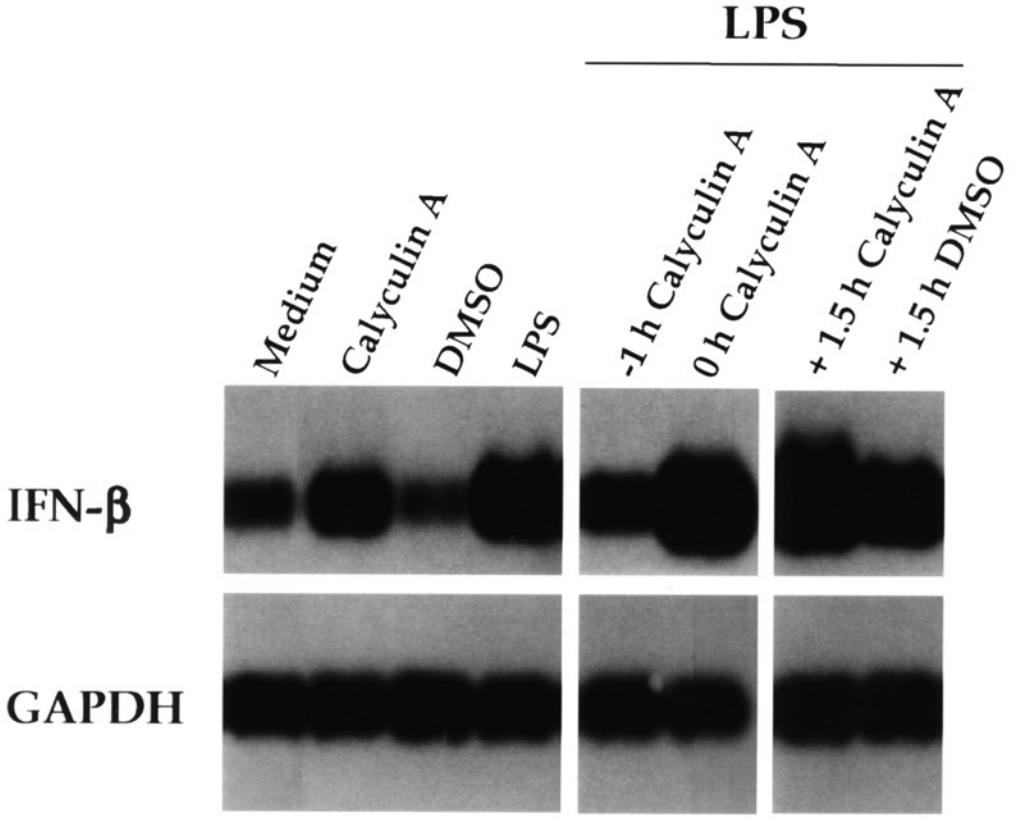

LPS
Medium
Calyculin A
DMSO
LPS
-1 h Calyculin A
0 h Calyculin A
+ 1.5 h Calyculin A
+ 1.5 h DMSO
IFN-β
GAPDH

Table V. Statistical Analysis of the Effect of Calyculin A on LPS-Induced Genes

Gene	Treatment[a]	Percent LPS Response (Range + 1 SEM)[b]
TNFα	LPS	100[d]
	Calyculin A	19.4[e] (13.5 - 27.7)
	LPS + Calyculin A	244[c] (171 - 350)
IL-1β	LPS	100[c]
	Calyculin A	8.8[d] (6.1 - 12.6)
	LPS + Calyculin A	99.6[c] (69.6 - 142)
IP-10	LPS	100[c]
	Calyculin A	1.3[e] (0.9 - 1.9)
	LPS + Calyculin A	16.1[d] (11.3 - 23.1)
IRF-1	LPS	100[c]
	Calyculin A	4.3[e] (3.0 - 6.2)
	LPS + Calyculin A	23.5[d] (16.4 - 33.6)
TNFR-2	LPS	100[c]
	Calyculin A	14.3[e] (10.0 - 20.5)
	LPS + Calyculin A	46.3[d] (32.4 - 66.3)

[a]Phosphorimager data was derived as described in the Materials and Methods from 3 independent Northern blot experiments like the one shown in Fig. 20. LPS (100 ng/ml) was added 1.5 hours prior to the addition of Calyculin A (25 nM).

[b]Data were analyzed by ANOVA on $\log_{10}$ transformed data to meet the assumptions of normality and homogeneity of variance. The data are expressed as the geometric mean and the numbers in parentheses represent the lower and upper values defined by 1 SEM. The LSD test was used for pairwise mean comparisons.

[c,d,e]Means are significantly different at the 5% level of significance of the LSD test.

Table VI: Effect of Calyculin A on LPS-induced TNF and IFN Production

Treatment	Bioactivity (U/ml)[a]	
	TNF	IFN
Medium only	< 80	<14.3
DMSO only	<80	<14.3
Calyculin A only	183	<15.7
LPS	27,667	87.0
-1h Calyculin A + LPS	100	17.7
+1.5 Calyculin A + LPS	19,795	158.7

[a]Results represent the geometric means of 3 - 5 separate experiments per treatment. For the TNF assay, the lowest detectable limit was 80 U/ml. For the IFN assay, the lowest detectable limit was 12.5 U/ml.

(Figures 20 and 21) did not correlate with an increase in the bioactivities.

One of the earliest detectable events after LPS signaling in macrophages is the tyrosine phosphorylation (TP) and activation of three MAP kinases, and in our system, these three proteins exhibit molecular weights of ~41.5, 43, and 47 kDa. We and others have shown that this event is maximal at 15 min after LPS stimulation and declines to baseline levels by 60-90 min (Weinstein *et al.*, 1991; Weinstein *et al.*, 1992, Dong *et al.*, 1993; Manthey *et al.*, 1992). Several studies utilizing TKI support the hypothesis that TP is required for LPS-induced TNF-α and IL-1β in murine macrophages and human monocytes (Beaty *et al.*, 1994; Shapira *et al.*, 1994; Novogrodsky *et al.*, 1994). Most recently, the results of a study using dominant negative inhibitors of *ras* and *raf-1* have extended this hypothesis, with the conclusion that the *ras/raf-1*/MEK/MAP kinase pathway is chiefly responsible for LPS-induced TNF-α mRNA (Geppert *et al.*, 1994). Because pre-treatment of macrophages with calyculin A prevented the LPS-induced expression of the TNF-α gene and all other genes examined, we sought to evaluate the effect of calyculin A on LPS-induced TP. As shown in Figure 22 (upper panel), LPS treatment of macrophages induced transient TP of the three proteins in the 40-45 kDa range identified previously as MAP kinases (Weinstein *et al.*, 1992; Han *et al.*, 1994). Treatment of macrophages with calyculin A alone also induced TP of three species with the same molecular weights as those seen with LPS; however, this induction failed to occur until 30-60 min after treatment (Figures 22, 23) and persisted for at least 8 h (Figure 23). In addition, calyculin A concurrently induced the tyr dephosphorylation of a ~61 kDa protein (Figure 23A,B;←). Simultaneous addition of calyculin A and LPS prolonged the tyr phosphorylated states of the three 40-45 kDa proteins (Figure 22).

Figure 22. Western blot analysis for the detection of tyrosine phosphoproteins (upper panel) and MAP kinases, *erk-1* and *erk-2* (lower panel). Cell lysates were prepared from C3H/OuJ peritoneal macrophages, as described in the Materials and Methods, after treatment with medium, 25 nM calyculin A, and 100 ng/ml LPS for the indicated times. Western blot analyses are shown after probing with antisera specific for tyrosine phosphoproteins (upper panel), and *erk-1* and *erk-2* (lower panel). The data shown is derived from a single representative experiment.

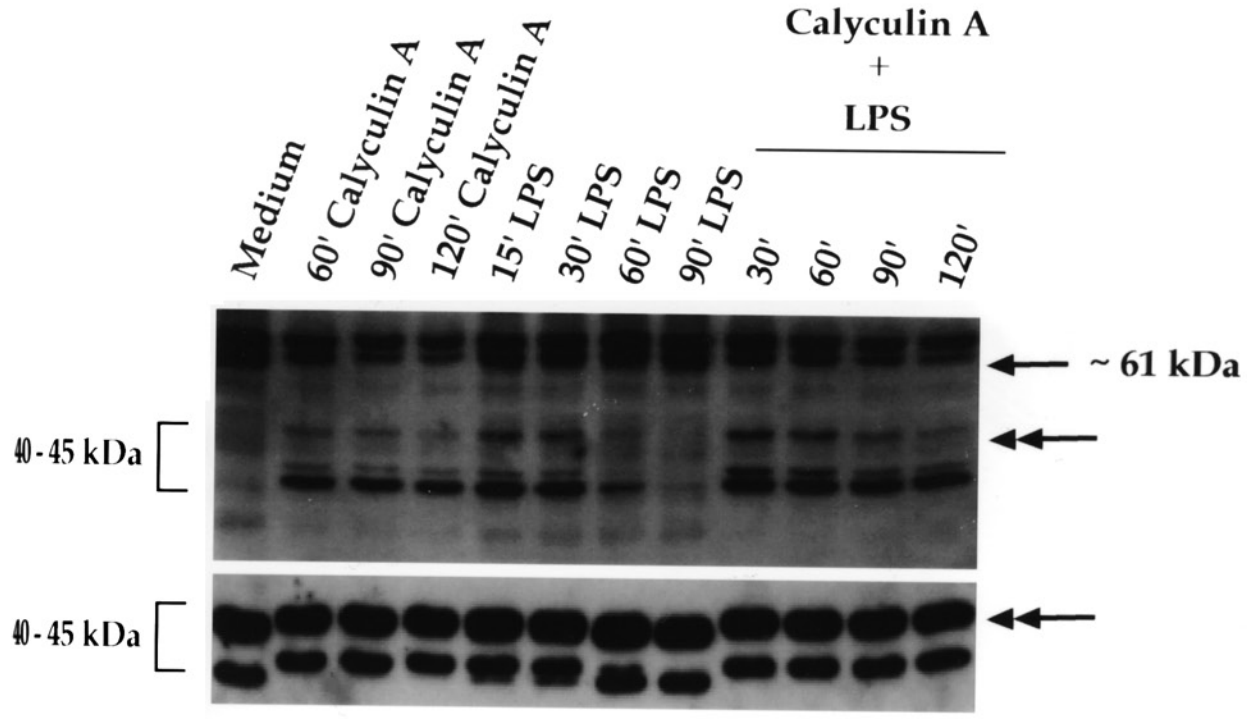
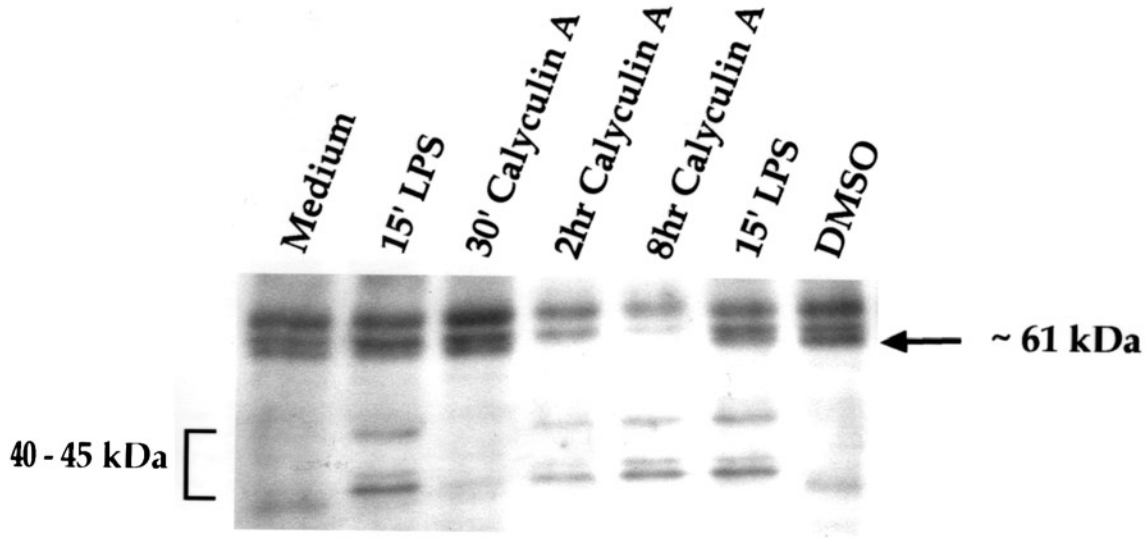

Calyculin A
+
LPS
Medium
60' Calyculin A
90' Calyculin A
120' Calyculin A
15' LPS
30' LPS
60' LPS
90' LPS
30'
60'
90'
120'
~ 61 kDa
40 - 45 kDa
40 - 45 kDa

Figure 23. Western blot analysis for the detection of tyrosine phosphoproteins. This blot was analyzed as described for Figure 22 (upper panel) for the detection of tyrosine phosphoproteins and has been enlarged to illustrate that at 30 min after calyculin A, one can barely detect the lowest MW MAP kinase species, yet all three species are detected as late as 8 hr after treatment. Also shown in this blot is the disappearance of a tyrosine phosphoprotein of ~61 kDa MW in cells treated with calyculin A for 2 or 8 hr.


Medium
15' LPS
30' Calyculin A
2hr Calyculin A
8hr Calyculin A
15' LPS
DMSO
~ 61 kDa
40 - 45 kDa

To confirm serologically that the three co-migrating tyrosine phosphorylated species induced by calyculin A were indeed the same MAP kinases induced by LPS, we stripped and reprobed the identical immunoblot with an antibody that is specific for *erk-1* and *erk-2*. Figure 22 (lower panel) illustrates that at 15 and 30 min after LPS stimulation, *erk-1* and *erk-2* exhibited slower mobilities than observed in medium-treated controls. After 60-90 min of LPS stimulation, a reappearance of the lower MW species, i.e., the dephosphorylated forms, occurred. The reappearance of the lower MW forms of *erk-1* and *erk-2* over time coincided exactly with the disappearance of TP shown in the upper panel (Note that the upper and lower panels of Figure 22 are superimposable using the double arrow as a reference point for alignment.) In calyculin A-treated macrophages, *erk-1* and *erk-2* also exhibited slower mobilities than observed in medium-treated controls and were indistinguishable from those present in the 15 min LPS-treated sample. In macrophages stimulated with both calyculin A and LPS, the higher MW forms of *erk-1* and *erk-2* predominated and the reappearance of the lower MW forms did not occur (in contrast to samples treated with LPS only), even at 120 min.

Effect of serine/threonine phosphatase inhibitor, okadaic acid. To assess further the role of ser/thr phosphatases in LPS-signal transduction, we also evaluated the dose- and time-dependent inhibition of cellular phosphatase activity by okadaic acid (OA). In contrast to calyculin A, which inhibits PP1 and PP2A with equal affinities, OA inhibits PP2A activity at concentrations significantly lower than required to inhibit PP1 (discussed below; Cohen and Cohen, 1989; Suganuma, *et al.*, 1990). C3H/OuJ peritoneal macrophages were cultured over time with various doses of okadaic acid or 100 ng/ml LPS, and

cell lysates were extracted and analyzed for phosphatase activity as described in the Materials and Methods. Based on preliminary dose response analyses (Table VII), 150 nM OA was used in all subsequent experiments, as this dose inhibited phosphatase activity to near maximal levels (i.e., a concentration ten-times higher was not substantially more effective), without compromising macrophage viability or adherence. The time-dependent inhibition of phosphatase activity in OA-treated macrophages as compared to LPS- and calyculin A-treated macrophages, is shown in Figure 24 (note that all data was derived from the same representative experiment depicted in Figure 19). In contrast to calyculin A, OA required approximately 8 h to inhibit phosphatase activity maximally (~ 59%).

Because two previous studies with okadaic acid suggested that OA alone stimulates the expression of TNF-α, IL-1β, IL-6 (Sung *et al.*, 1992), and IP-10 (Tebo *et al.*, 1994) genes in macrophages, we expanded our panel of LPS-inducible genes to include TNF-α, IL-1β, IFN-β, IP-10, IRF-1, TNFR-2, IRF-2, ICSBP, IL-6, IL-10, and IL-12 (p40) (i.e., the inducible chain of IL-12). C3H/OuJ macrophages were treated with medium only, OA only (150 nM), LPS (100 ng/ml), or LPS plus OA, and total cellular RNA was collected at various times. Prior to 12 h of treatment there was no detectable effect of OA on the expression of any gene examined (data not shown). The results of a 12 h time point from a representative experiment (n = 3) are shown in Figure 25 (Northern blot) and Figure 26 (RT-PCR). Confirming and extending previous observations, treatment of macrophages with OA only for 12 h resulted in readily detectable levels of TNF-α, IL-1β, IP-10 (Figure 25), IFN-β, and IL-6 (Figure 26) mRNA. Treatment with OA alone also resulted in a slight increase in basal levels of IRF-1 mRNA (<3 fold), and a slight decrease in basal

Table VII: Sensitivity of Macrophage Phosphatase Activity to Okadaic Acid[a]

Concentration of Okadaic Acid	% Phosphatase Activity[b]
15 nM	93 ± 7.69
150 nM	59 ± 4.86
1500 nM	42 ± 3.36

[a]Macrophages were treated as described in the Materials and Methods with the indicated concentrations of okadaic acid for 8 - 9 h.

[b]Data represent the arithmetic mean ± SEM of 3 separate experiments. The data are expressed as percent of the medium-treated controls for each experiment.

Figure 24. Phosphatase activity in macrophages treated with LPS or okadaic acid. Whole cell lysates of peritoneal exudate macrophages from C3H/OuJ mice were prepared and assayed for phosphatase activity as described in the Materials and Methods. Phosphatase activity is expressed as a percent of medium-treated controls and the phosphatase activity of the control group varied < 5% over time. The data shown is derived from a single experiment that is respresentative of 3 separate experiments.

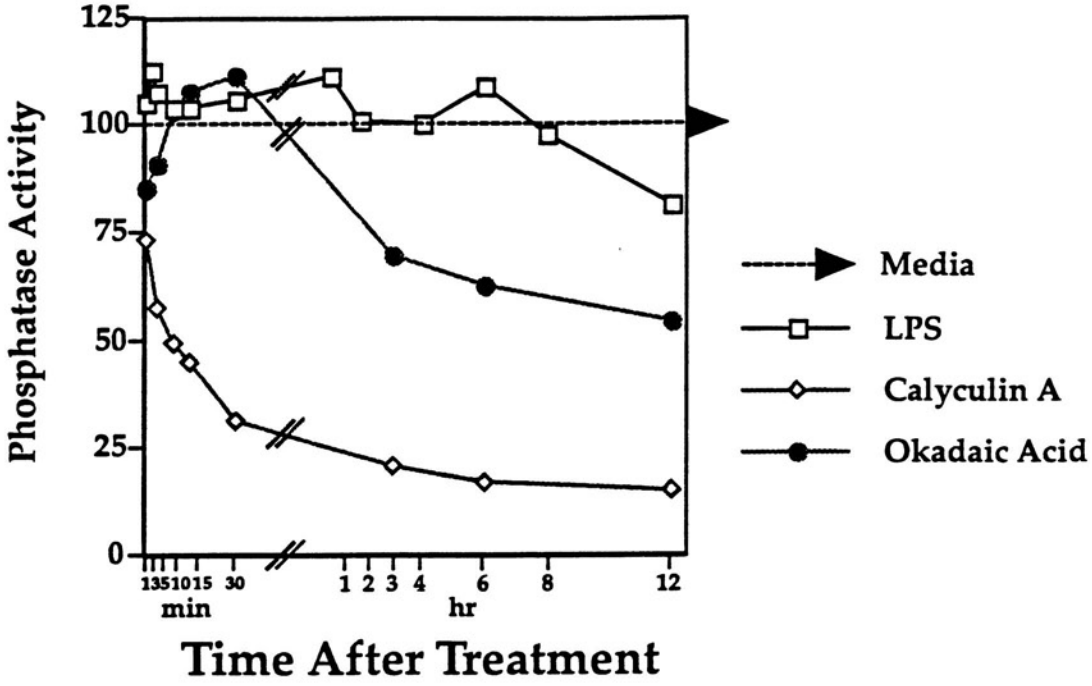

Phosphatase Activity
125
100
75
50
25
0
1 3 5 10 15 30
min
1 2 3 4 6 8 12
hr
Time After Treatment
Media
LPS
Calyculin A
Okadaic Acid

Figure 25. Northern blot analysis of TNF-α, IL-1β, IP-10, and GAPDH gene expression in C3H/OuJ macrophages treated for 12 h as indicated with medium only, 150 nM okadaic acid (OA), and/or 100 ng/ml LPS. The data shown is derived from a single experiment that is representative of 3 separate experiments.

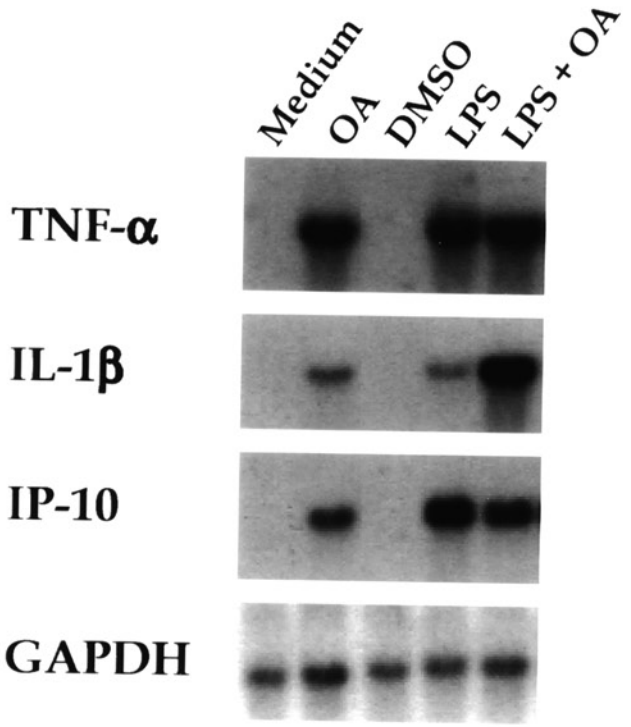
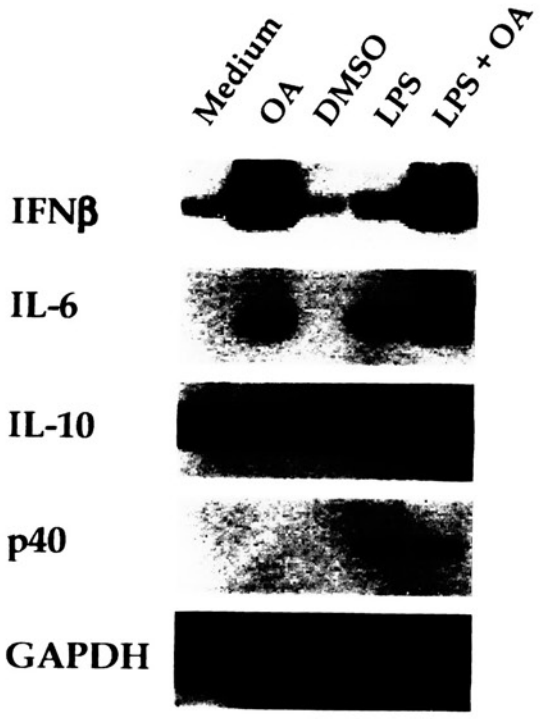

Medium
OA
DMSO
LPS
LPS + OA
TNF-α
IL-1β
IP-10
GAPDH

Figure 26. RT-PCR analysis of IFN-β, IL-6, IL-10, p40 and GAPDH gene expression in C3H/OuJ macrophages treated for 12 h as indicated with 150 nM okadaic acid, and/or 100 ng/ml LPS. The data shown is derived from a single experiment that is representative of 3 separate experiments.


Medium
OA
DMSO
LPS
LPS + OA
IFNβ
IL-6
IL-10
p40
GAPDH

levels of IRF-2 and ICSBP mRNA (~2 fold), while no effect was detected in basal expression of TNFR-2, IL-10 and IL-12 (p40) mRNA. (Figure 26; data not shown). To determine if OA also stimulated not only the expression of TNF-α and IFN-β mRNA, but also the secretion of TNF and IFN, bioassays were performed on macrophage culture supernatants collected at the same time as described for the mRNA analyses. As shown in Table VIII, bioactivities for TNF and IFN were detected in supernatants from macrophages treated with OA alone.

The effect of OA on LPS-inducible gene expression was also assessed. Although 12 h is beyond the peak of LPS-induced expression of most of the genes in this panel (Tannenbaum *et al.*, 1988; Manthey *et al.*, 1992; Barber *et al.*, 1994), a significant level of steady-state mRNA was nonetheless measurable for each gene. When OA was present concurrently with LPS, decreased expression of IRF-1, TNFR-2, and IL-12 (p40) genes resulted, while no effect was observed on the LPS-induced expression of TNF-α, IP-10, IL-6, IRF-2, and ICSBP genes (Figures 25 and 26; data not shown). In contrast, the combination of LPS and OA led to an enhancement of the LPS-induced expression of IL-1β, IFN-β, and IL-10 mRNA (Figures 25 and 26). However, the enhanced expression of IFN-β mRNA was essentially the same level of expression induced in response to OA alone (Figure 26). Analysis of the cumulative data derived from 3 separate experiments is shown in Table IX, and confirms the observations depicted in the representative experiment shown in Figures 25 and 26.

Thus far, our studies indicate that OA stimulates an inflammatory response in peritoneal macrophages. Because OA has been shown to be a potent tumor promoting agent (Suganuma *et al.*, 1988), and macrophage

Table VIII: Effect of Okadaic Acid of TNF and IFN Production

Treatment	Bioactivity (U/ml)[a]	
	TNF	IFN
Medium only	<80	<12.5
DMSO only	<80	<12.5
Okadaic Acid only	16,218	251

[a]Results represent the geometric means of 3 - 5 separate experiments per treatment. For the TNF assay, the lowest detectable limit is 80 U/ml. For the IFN assay, the lowest detectable limit is 12.5 U/ml.

Table IX. The Effect of Okadaic Acid on LPS-Induced Gene Expression

Gene	Treatment[a]	Percent Maximum LPS Response (Range + 1 SEM)[b]
IL-1β	LPS	100
	LPS + OA	533.3 (398 - 714)
IFN-β	LPS	100
	LPS + OA	6,309.6 (1018.6 - 39,084.1)
IL-10	LPS	100
	LPS + OA	1,422.3 (889.2 - 2275.1)
IRF-1	LPS	100
	LPS + OA	45.4 (42.1 - 49)
TNFR-2	LPS	100
	LPS + OA	34.4 (29.5 - 40.2)
p40	LPS	100
	LPS + OA	54.2 (30.6 - 95.9)

---

[a]Macrophages were treated as described in the Materials and Methods with the indicated concentrations of okadaic acid for 12 h.

[b]The data are expressed as the geometric mean and the numbers in parentheses represent the lower and upper values defined by 1 SEM, and were derived from 3 separate experiments.

tumoricidal activity has been linked to the production of nitric oxide ($NO^{\bullet}$), we next sought to examine the effect of OA on $NO^{\bullet}$ production. It has been well established that activation of murine macrophages to produce $NO^{\bullet}$ and to become optimally tumoricidal requires two signals (Lorsbach *et al.*, 1993). Typically, a synergistic combination of LPS and IFN-γ is used to achieve this effect (reviewed in Nathan, 1992). $NO^{\bullet}$ is a very unstable radical, and is rapidly converted to the stable metabolite, NO_2^-, *in vitro*. Therefore, NO_2^- was measured in supernatants derived from macrophages treated with LPS, IFN-γ, OA, or various combinations of the three. The results, shown in Figure 27, indicate that OA alone did not induce the production of $NO^{\bullet}$; however, the low amounts of NO_2^- produced in response to either LPS or IFN-γ were decreased in the presence of OA. Furthermore, OA inhibited NO_2^- produced in response to the synergistic combination of LPS and IFN-γ.

Previous studies indicate that although the majority of inducible $NO^{\bullet}$ occurs at the level of transcription, some post-transcriptional regulation may occur as well (reviewed in Xie and Nathan, 1994). Therefore, we analyzed the effect of OA on the expression of the iNOS (described above) and IRF-1 (a positive regulator of iNOS transcription; Matsuyama et al., 1993; Kamijo et al., 1994; Martin et al., 1994) genes. The results are shown in Figure 28. Consistent with the results obtained by measuring NO_2^-, LPS, IFN-γ, and to a lesser extent OA, stimulated iNOS gene expression, whereas a combination of LPS and IFN-γ induced much greater levels. OA, when added simultaneously with LPS + IFN-γ, resulted in a dose dependent decrease in iNOS mRNA levels. The effects of OA on IRF-1 gene expression, in this system, showed a parallel pattern. Increasing the dose of IFN-γ, from 5 U/ml to 10 U/ml, was not able to overcome the OA-mediated inhibition of either gene.

Figure 27. Effect of okadaic acid on $NO^{\bullet}$ production in activated macrophages. C3H/OuJ macrophages were treated as indicated with LPS (100 ng/ml), IFN-γ (5 U/ml), and/or okadaic acid (150 nM). The data are expressed as the concentration NO_2^- detected in each sample as described in Materials and Methods, and represent the arithmetic means derived from 3 separate experiments.

Effect of Okadaic Acid on Macrophage Production of $NO^{\cdot}$

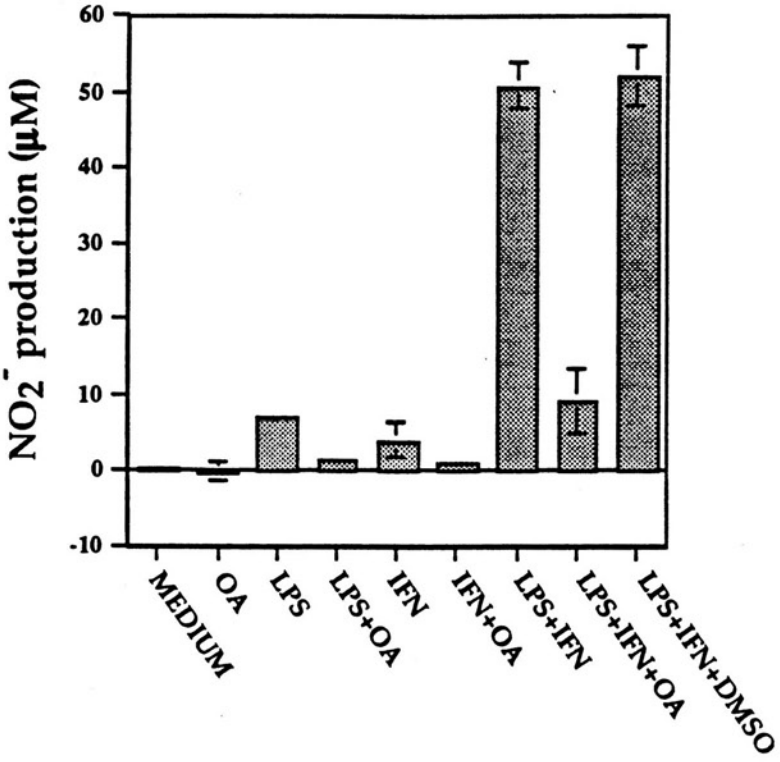
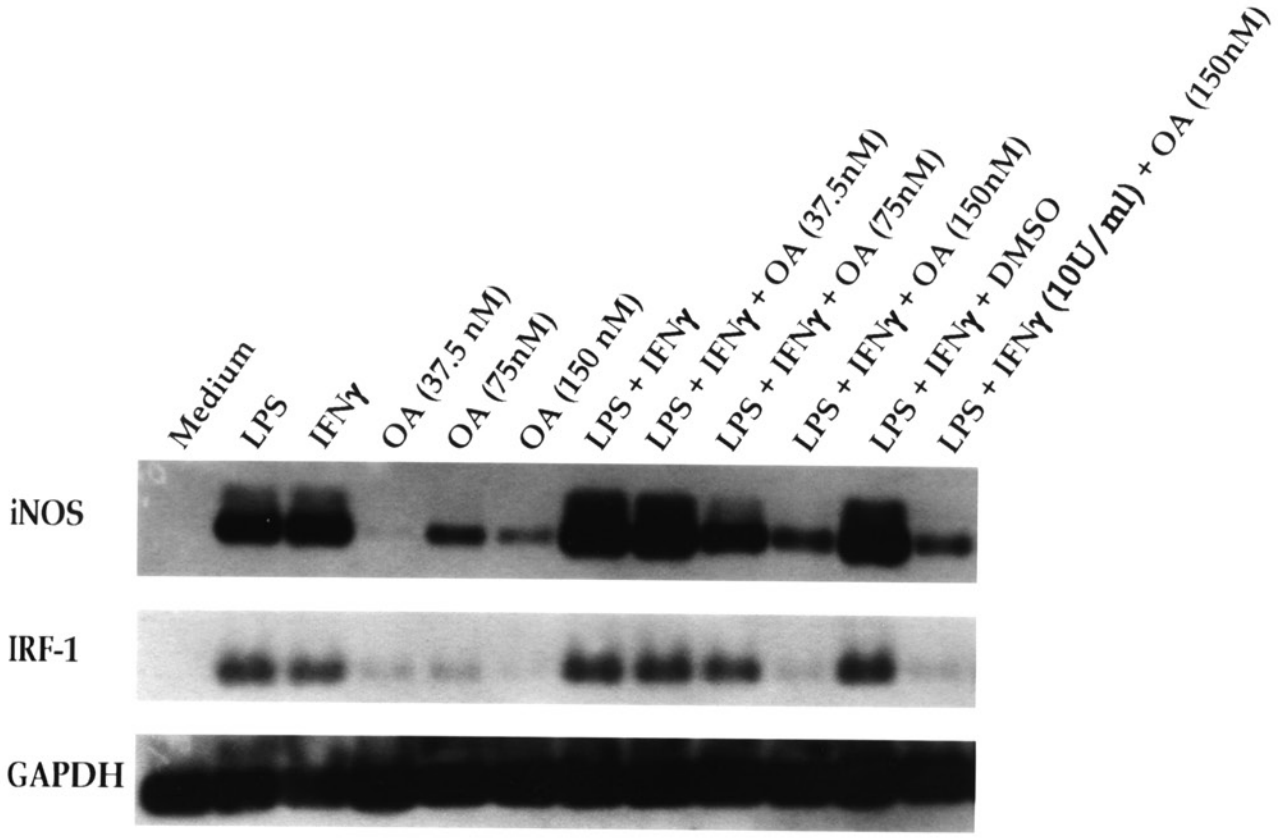


Figure 28. RT-PCR analysis of iNOS, IRF-1, and GAPDH gene expression in C3H/OuJ macrophages treated as indicated with 150 nM okadaic acid, 100 ng/ml LPS and/or 5 U/ml IFN-γ (unless otherwise noted). The data shown is derived from a single experiment that is representative of 3 separate experiments.


Medium
LPS
IFNγ
OA (37.5 nM)
OA (75nM)
OA (150 nM)
LPS + IFNγ
LPS + IFNγ + OA (37.5nM)
LPS + IFNγ + OA (75nM)
LPS + IFNγ + OA (150nM)
LPS + IFNγ + DMSO
LPS + IFNγ (10U/ml) + OA (150nM)
iNOS
IRF-1
GAPDH

ANALYSIS OF THE POTENTIAL ROLE OF THE CERAMIDE-ACTIVATED PATHWAY IN LPS SIGNALING LEADING TO GENE EXPRESSION

Lipid second messengers are gaining recognition as important mediators of extracellular signals. One such lipid, ceramide, has recently been appreciated as a lipid second messenger of TNF-α, IL-1β, and IFN-γ signaling pathways and is generated from membrane sphingomyelin by the action of sphingomyelinase (SMase) (reviewed in Kolesnick and Golde, 1994). Ceramide, in turn, mediates the activation of membrane-associated ceramide-activated protein kinase (CAK) and ceramide-activated protein phosphatase (Liu *et al.*, 1994; Dobrowsky and Hannun, 1993). A recent study illustrated a striking structural similarity between a portion of the lipid A region of LPS and ceramide and demonstrated the ability of LPS to activate CAK, in a manner that was both CD14-dependent and enhanced by LBP, but ceramide-independent (Joseph *et al.*, 1994). Thus, the authors suggested that LPS stimulates cells by mimicking the second messenger function of ceramide.

As presented above, the ability to respond to LPS has been linked genetically to the *Lps* locus on mouse Chromosome 4 (Watson *et al.*, 1978). To clarify the potential role of the ceramide pathway in LPS signaling, we sought to determine if the ability to respond to ceramide was influenced by allelic expression of the *Lps* locus. To this end, we analyzed the expression of a panel of LPS-inducible genes in macrophages derived from LPS-responsive (Lps^n) C3H/OuJ and LPS-hyporesponsive (Lps^d) C3H/HeJ mice, following stimulation with LPS, ceramide, and SMase.

Influence of *Lps* allelic expression on the activation of the ceramide pathway. To assess the influence of *Lps* allelic expression on the ability to respond to

ceramide, peritoneal macrophages from C3H/OuJ (Lps^n) and C3H/HeJ (Lps^d) mice were cultured for 2, 4.5, or 6 hr with 100 ng/ml LPS, various concentrations of cell-permeable analogs of ceramide (C_2, C_6, C_{16}), or SMase. Total RNA was extracted and subjected to Northern analysis of TNF-α, IL-1β, IRF-1, TNFR-2, and GAPDH mRNA. Figure 29 shows the results of a single representative experiment in which C_2-ceramide was used to stimulate macrophages for 4.5 hr. Consistent with their *Lps* allelic designations, C3H/OuJ, but not C3H/HeJ macrophages responded to protein-free LPS to induce the expression of TNF-α, IL-1β, IRF-1, and TNFR-2 genes. Similarly, C_2-ceramide (as low as 6.25 μM; data not shown) stimulated C3H/OuJ macrophages to increase the expression of each gene, while again, C3H/HeJ macrophages failed to respond. The identical pattern of results was obtained when C6- and long-chain C16-ceramides were used to stimulate macrophages from both strains (data not shown). In contrast, C3H/HeJ macrophages responded to a protein-rich preparation of RaLPS, as previously reported (Manthey *et al.*, 1994). We next assessed the ability of C3H/OuJ and C3H/HeJ to respond to naturally-derived ceramide (i.e., generated from macrophage membrane sphingomyelin by the action of SMase). Figure 30 shows the results of a single representative experiment (n = 4) in which exogenous SMase was used to stimulate macrophages derived from both strains of mice. Again, the C3H/OuJ macrophages responded to SMase to increase the expression of TNF-α mRNA, while there was no TNF-α mRNA induction observed in SMase-stimulated C3H/HeJ macrophages. In constrast, C3H/HeJ macrophages responded to protein-rich, butanol-extracted LPS (But-LPS), as previously reported (Hogan *et al.*, 1988). In no experiment (n = 5 for ceramides; n = 4 for SMase) was there any suggestion of gene induction in the

Figure 29. Northern blot analysis of C3H/OuJ and C3H/HeJ macrophages stimulated with 100 ng/ml protein-free LPS (LPS), 10 μg/ml protein-rich RaLPS, or the indicated concentrations of C2-ceramide. The data shown is derived from a single experiment that is representative of 5 separate experiments.

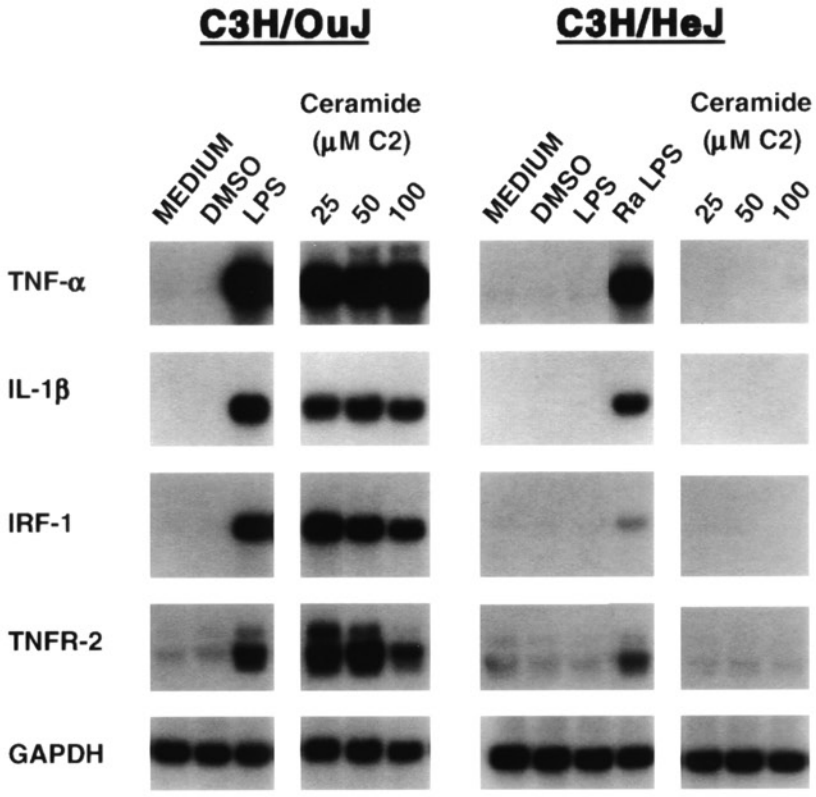
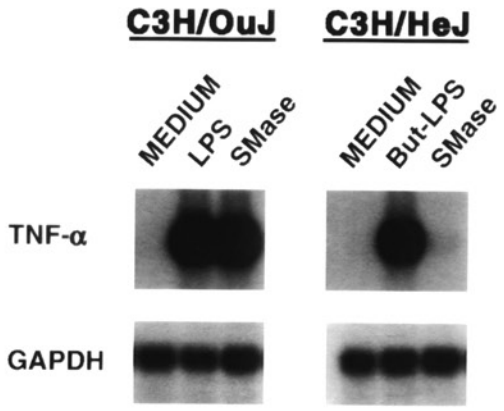

C3H/OuJ
C3H/HeJ
Ceramide (μM C2)
MEDIUM
DMSO
LPS
25
50
100
Ra LPS
TNF-α
IL-1β
IRF-1
TNFR-2
GAPDH

Figure 30. Northern blot analysis of C3H/OuJ and C3H/HeJ macrophages stimulated with 100 ng/ml protein-free LPS (LPS), 10 μg/ml protein-rich But-LPS, or 1 U/ml SMase. The data shown is derived from a single experiment that is representative of 4 separate experiments.


C3H/OuJ
C3H/HeJ
MEDIUM
LPS
SMase
MEDIUM
But-LPS
SMase
TNF-α
GAPDH

C3H/HeJ macrophages stimulated with any of the ceramides (6.25 - 100 μM; 4.5 h) or SMase (0.06 - 2 U/ml; 2 - 6 h).

Comparison of LPS- and ceramide-inducible gene expression in C3H/OuJ macrophages. The above results imply that stimulation of macrophages by LPS and ceramide requires expression of the *Lps*^{n} allele. If the signaling pathways elicited by ceramide and LPS are identical, then the pattern of gene expression resulting from ceramide stimulation should mimic that induced by LPS. Conversely, if the pathways elicited by ceramide and LPS diverge (i.e., after the requirement for the *Lps*^{n} gene product), then a different pattern of gene expression may result. A visual assessment of the data shown in Figure 29 suggested that the levels of expression of TNF-α, IL-1β, IRF-1, and TNFR-2 genes are fairly comparable in LPS- or ceramide-stimulated C3H/OuJ macrophages. However, not all genes examined exhibited comparable levels of inducibility. Figure 31 shows a comparison of TNF-α and IP-10 gene expression in C3H/OuJ macrophages treated with LPS or various doses of C_2-C_6-, or C_{16}-ceramide. In contrast to the expression of TNF-α, IL-1β, IRF-1, and TNFR-2 genes, IP-10 gene expression is sub-optimally induced by the ceramides at all doses examined when compared to LPS. Analysis of the pooled data from 5 - 6 separate experiments is shown in Table X, which expresses the data as the percentage of the LPS-induced response for each gene. Ceramide-induced levels of IL-1β and TNFR-2 gene expression were generally higher than the levels induced by 100 ng/ml LPS, whereas the levels of TNF-α, IRF-1, and IRF-2 gene expression were quite similar. In contrast, the levels of IP-10 and ICSBP gene expression induced by ceramide were consistently and substantially lower than those induced by LPS at 4.5 h.

Figure 31. Representative PhosphorImager analysis of TNF-α and IP-10 gene expression in C3H/OuJ macrophages stimulated with the indicated doses of C_2-, C_6-, or C_{16}-ceramide for 4.5 h, graphed as percent of the maximum LPS-induced gene expression in each experiment (100%).

Ceramide-Induced TNF-α and IP-10 Gene Expression

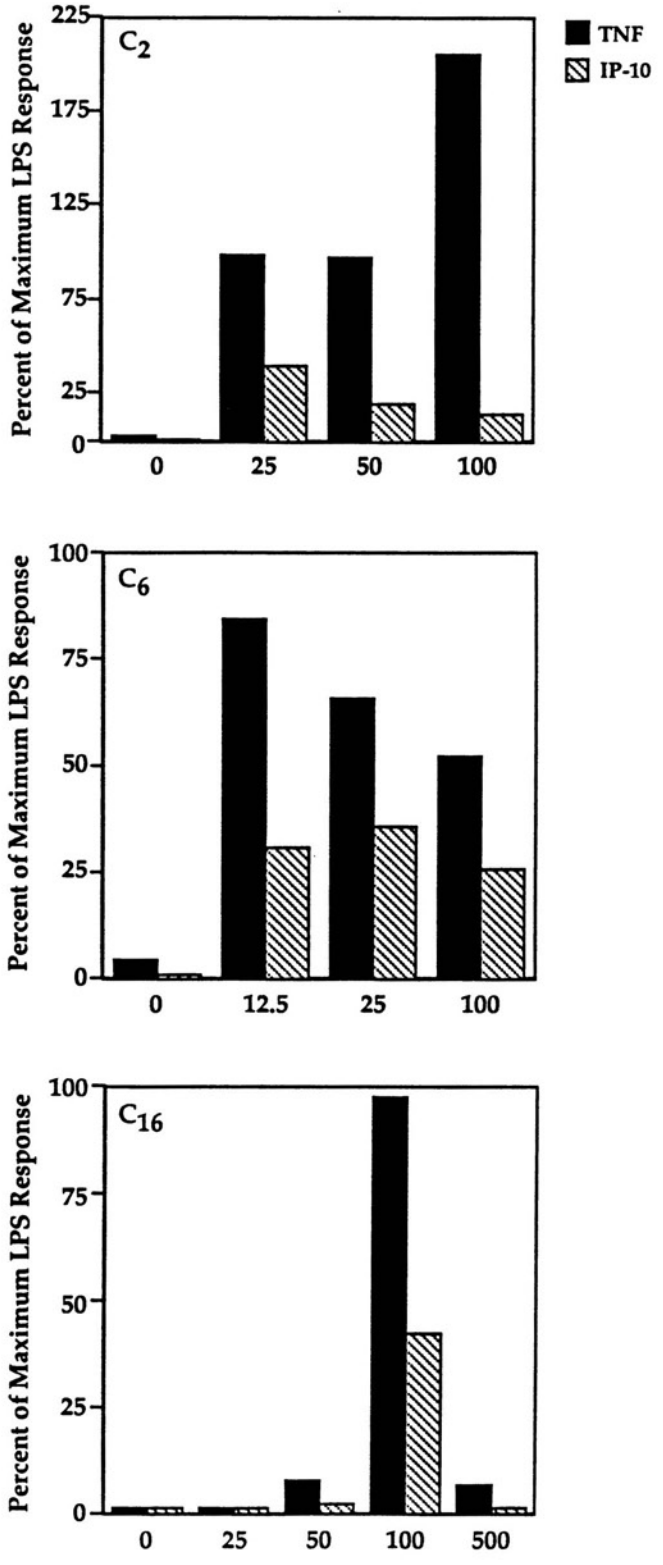


Table X: Comparison of SMase- and LPS-induced Gene Expression

Gene	Treatment[a]	Percent of Maximum LPS Response (Range + 1 SEM)[b]
TNFα	LPS	100
	Ceramide	119.1 (104.7 - 135.5)
IRF-1	LPS	100
	Ceramide	85.5 (78.2 - 93.5)
IRF-2	LPS	100
	Ceramide	85.5 (76.6 - 95.5)
IL-1β	LPS	100
	Ceramide	146.5 (124.5 - 172.6)
TNFR-2	LPS	100
	Ceramide	156.7 (129.4 - 189.7)
IP-10	LPS	100
	Ceramide	28.4 (20.6 - 39.4)
ICSBP	LPS	100
	Ceramide	36.9 (25.6 - 53.2)

[a]PhosphorImager data was derived as described in the Materials and Methods from 5 or 6 independent Northern blot experiments like the one shown in Figure 29.

[b]The data are expressed as a percent of the LPS response (100 ng/ml) at 4.5 h for each gene and were derived from the ceramide (C_2, C_6, and C_{16}) concentrations that resulted in the maximally induced level of expression of each gene in each experiment. The results are expressed as the geometric mean and the numbers in parentheses represent the lower and upper values defined by 1 SEM.

Similar results were obtained when SMase was used to stimulate the macrophages, and despite high LAL activities present in certain SMase preparations (ranging between 1.1 - 12.0 ng/ml LPS in 1 U/ml SMase), IP-10 gene expression was always less than the maximum LPS response when tested between 2 and 6 h, with doses of SMase as high as 2 U/ml (data not shown). This is in striking contrast to the ceramide experiments where no lots contained detectable LAL activity (see Materials and Methods). Therefore, the data derived from SMase stimulation was not included in Table X because of the potentially confounding and variable presence of endotoxin contamination in the SMase preparations used in the time-course and dose-response experiments. However, the ability of C3H/OuJ macrophages to secrete TNF and IFN in response to a SMase lot that contained only trace levels of LPS (0.03 ng/ml LPS in 1 U/ml SMase, as assessed by LAL) was also measured. The data shown in Table XI, indicates that TNF, but not IFN, was detected in supernatants derived from C3H/OuJ macrophages treated with SMase. Even after 19 h of stimulation, with as much as 2 U/ml SMase, no IFN bioactivity was observed (data not shown).

Effect of calyculin A on SMase-induced gene expression. The data presented in the second section of my Results support the hypothesis that ser/thr phosphatases are involved in the regulation of the LPS signaling pathway (above). Therefore, the involvement of ser/thr phosphatases in the regulation of the ceramide signaling pathway was also assessed. As described above, pretreatment of macrophages with 25 nM calyculin A for one hour inhibits the expression of every LPS-inducible gene examined (Figures 20, 21). To evaluate the dependence of ceramide signaling on ser/thr phosphatases, total cellular RNA or supernatants derived from macrophages that were pre-

Table XI: Comparison of TNF-α and IFN Production Induced by LPS or Smase[a]

Treatment	TNF-α (pg/ml)	IFN Bioactivity (U/ml)
Medium only	< 35	<14.3
LPS (100 ng/ml)	1,910	119
SMase[b] (1 U/ml)	1,600	<14.3

[a]Supernatants were collected from macrophages that were stimulated for 4.5 h, with either LPS or SMase and analyzed for the production of TNF or IFN. Results represent the geometric means of 4 - 6 separate experiments per treatment. For the TNF ELISA, the lowest concentration of rTNF included on the standard curve was 35 pg/ml. For the IFN bioassay, the lowest detectable limit was 12.5 U/ml.

[b]The SMase used in each of these experiments contained 0.03 ng/ml LPS as measured by LAL.

treated for 1 h with medium or 25 nM calyculin A prior to stimulation with either LPS or SMase, were analyzed for TNF-α mRNA levels or TNF protein. The results of an experiment that is representative of four similar experiments are shown in Table XII. Consistent with the results presented above, pre-treatment with calyculin A inhibited TNF production in LPS-stimulated macrophages. Similarly, TNF produced in response to SMase was inhibited by pre-treatment with calyculin A. These results reflect the inhibition observed at the RNA level for all genes examined (TNF-α, IL-1β, IRF-1, and TNFR-2; data not shown).

Comparison of LPS- and SMase- inducible gene expression in endotoxin-tolerized C3H/OuJ macrophages. As described above, *Lps*^{n} macrophages pre-treated with endotoxin, exhibit an LPS-hyporesponsive phenotype following subsequent challenge with LPS. If LPS stimulates cells by mimicking the second messenger function of ceramide, as suggested by Joseph *et al.* (1994), then it might be expected that macrophages pre-treated with endotoxin would be tolerant to ceramide, and macrophages pre-treated with ceramide would be tolerant to challenge with LPS. To address this issue, macrophages were pre-treated as decsribed in the Materials and Methods with medium only, LPS (100 ng/ml), or SMase (1 U/ml), and subsequently challenged with either medium alone, LPS (100 ng/ml), or SMase (1 U/ml). Supernatants were collected and analyzed for the production of TNF. The data shown in Table XIII indicate that macrophages "tolerized" to LPS produced very low levels of TNF in response to challenge with LPS or SMase. Conversely, macrophages pre-treated with SMase were tolerant to neither LPS nor SMase.

Table XII: Effect of Calyculin A on TNF Production Induced by LPS or SMase[a]

	TREATMENT		
PRE-TREATMENT	Medium	LPS	SMase
Medium	<35	2,125	2,053
Calyculin A (-1 h)	<35	<35	<35

[a]Supernatants were collected from macrophages that were pre-treated for 1 h with medium only or with calyculin A prior to stimulation with medium, LPS, or SMase (for 4.5 h), and analyzed for the production of TNF-α. For the TNF ELISA, the lowest concentration of rTNF included on the standard curve was 35 pg/ml. The SMase used in this experiment contained 0.03 ng/ml LPS as measured by LAL. The data are representative of 4 separate experiments.

Table XIII: TNF Production in Macrophages Pre-treated with LPS or SMase

	CHALLENGE		
PRE-TREATMENT	Medium	LPS	SMase
Medium	<35	2,065	1,782
LPS	<35	<35	127
SMase	<35	2,291	1,349

Macrophages were pre-treated with medium only, LPS, or SMase for 19 h as described in the Materials in Methods. Supernatants collected from macrophages after subequent challenge with medium only, LPS, or SMase for 4.5 h, were analyzed for the production of TNF. For the TNF ELISA, the lowest concentration of rTNF included on the standard curve was 35 pg/ml. The SMase used in each of these experiments contained 0.03 ng/ml LPS as measured by LAL. Results represent the geometric means of 3 separate experiments.

DISCUSSION

Nuclear Regulation of LPS-inducible Gene Expression: Analysis of IRF DNA binding proteins in LPS-induced signaling

LPS-hyporesponsive models have been invaluable tools used to elucidate the cellular mechanisms that are involved in macrophage signal transduction, gene expression, differentiation, and activation (Manthey *et al.*, 1992; Salkowski *et al.*, 1992; Dong *et al.*, 1993). In the simplest model, LPS stimulation of macrophages begins with signal transduction across the cell membrane that triggers a multitude of intracellular biochemical events, some of which result in the activation and nuclear mobilization of transcription factors that regulate LPS-inducible gene expression. Ultimately, the cellular LPS-responsive or LPS-hyporesponsive phenotype must reflect a corresponding molecular phenotype directed by transcriptional activators and repressors. The primary goals of these experiments were to characterize the LPS-inducibility of the genes that encode the ISRE-binding transcription factors IRF-1, IRF-2, and ICSBP, and to compare the expression of these genes in two distinct models of LPS-hyporesponsiveness.

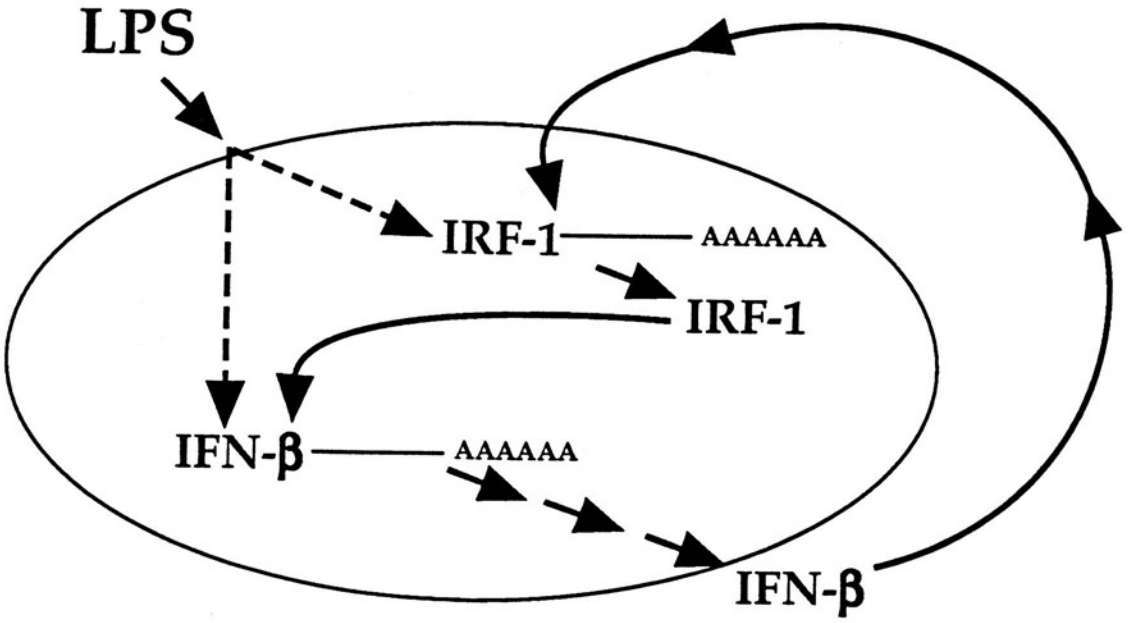
Initial studies were focused on a well-characterized, genetic model of LPS-hyporesponsiveness. From the data provided in Figure 1, Lps^n macrophages were observed to express higher basal levels of the transcriptional activator IRF-1 mRNA, while Lps^d macrophages express higher basal levels of the transcriptional repressor IRF-2 mRNA. In addition, consistently higher basal levels of IFN-β mRNA were found in Lps^n macrophages, compared to Lps^d macrophages, thus providing molecular evidence to support previous protein neutralization studies in which anti-

IFN-α/β antibodies rendered *Lps*^{n} macrophages phenotypically like *Lps*^{d} macrophages with respect to FcγR expression and virus susceptibility (Vogel *et al.*, 1986; Gessani *et al.*, 1987; Vogel *et al.*, 1987).

The data gathered from the *Lps*^{n} and *Lps*^{d} macrophages may be considered in the context of the positive feedback model of IFN-β gene regulation proposed by Harada *et al.* (1989) to describe the differential basal level gene expression exhibited by LPS-responsive and LPS-hyporesponsive phenotypes. In this model, depicted in Figure 32, IFN-β induced in *Lps*^{n}, but not *Lps*^{d} macrophages, by exposure to LPS *in vivo*, may act in an autocrine fashion to increase the basal levels of IRF-1 mRNA in *Lps*^{n} macrophages. In turn, IRF-1 would sustain additional IFN-β steady-state mRNA levels. Such a situation would favor an increased ratio of IRF-1/IRF-2 in *Lps*^{n} cells, and perhaps accounts for some of the observed functional differences between *Lps*^{n} and *Lps*^{d} macrophages. As discussed above, treatment of *Lps*^{d} macrophages with IFN-α normalizes some of the functional differences associated with macrophage differentiation. In this study, IFN-α was found stimulate *Lps*^{d} macrophages to undergo a rapid transition in IRF molecular phenotype (Figure 4). Within one hour, IRF-1 mRNA levels were increased (~40 fold) in the absence of a corresponding increase in IRF-2 mRNA. Since the relative fold induction of IRF-1 mRNA far exceeded that of IRF-2 mRNA throughout the time course, the phenotype of the IFN-α-treated C3H/HeJ macrophage correlated with an early and sustained increase in the ratio of IRF-1/IRF-2 gene expression.

IRF family gene expression was also analyzed in *Lps*^{n} macrophages stimulated with exogenous LPS. As with many LPS-inducible genes (Tannenbaum *et al.*, 1988), accumulation of IRF-1 mRNA in *Lps*^{n}

Figure 32. Model depicting a potential autocrine loop of IRF-1 and IFN-β gene expression in *Lps*^{n} macrophages


LPS
IRF-1
AAAAAA
IRF-1
IFN-β
AAAAAA
IFN-β

macrophages occurs rapidly (an increase in IRF-1 mRNA is detectable within 30 minutes of LPS stimulation; data not shown), while an increase in IRF-2 and ICSBP mRNA levels is not detected until ~4 hours. The lag time prior to the increase in IRF-2 mRNA levels compared to IRF-1 mRNA is consistent with recent data that suggest a role for IRF-2 in the negative feedback regulation of gene expression resulting from potent bioactive stimuli such as LPS (Hayes *et al.*, 1993). The increase in IRF-1 gene expression (40-45 fold) far exceeds that of IRF-2 and ICSBP genes (4-6 fold), which may be an important compensatory mechanism for IRF-1-mediated functions, since the IRF-1 protein has a much shorter half-life, i.e., ~30 min, than that of IRF-2 or ICSBP ($t_{1/2} \geq 8$ hr; Harada *et al.*, 1990; Watanabe *et al.*, 1991; Politis *et al.*, 1994). In response to LPS, the accumulation of all three species of mRNA was prolonged, perhaps due to the production of LPS-inducible cytokines such as TNF-α, IL-1β, IL-6, and IFN-$\alpha/\beta/\gamma$, all of which have been shown to augment IRF-1, IRF-2 and/or ICSBP (IFN-γ only) gene expression (Fujita *et al.*, 1989; Harada *et al.*, 1989; Driggers *et al.*, 1990; Pine *et al.*, 1990; Abdollahi *et al.*, 1991; Tanaka *et al.*, 1992). However, IRF-1 mRNA accumulates as a direct consequence of LPS stimulation, as evidenced by its insensitivity to cycloheximide (Fig. 4). Interestingly, cycloheximide alone increases steady-state levels of IRF-1 mRNA that are superinduced in the presence of LPS. This suggests that LPS-inducible IRF-1 gene expression may be regulated, in part, at the level of mRNA stability. Two DNA sequence motifs that may confer mRNA instability have been found in the 3' noncoding region of the IRF-1 cDNA sequence (Miyamoto *et al.*, 1988).

IRF family gene expression was also analyzed in normally LPS-responsive macrophages that have been rendered transiently hyporesponsive

to a second exposure of LPS (Virca *et al.*, 1989). At the time of LPS "challenge," all IRF mRNA levels were still above basal levels. Thus, the pattern of IRF-1 and ICSBP gene expression in this LPS-hyporesponsive model differs from the *Lps*^{d} model. Re-stimulation of endotoxin-tolerized macrophages with LPS failed to re-induce IRF-1, IRF-2 or ICSBP mRNA levels. As reported previously, IP-10 gene expression was very low in tolerized cells and was not re-induced upon LPS re-stimulation (Henricson *et al.*, 1993). Minimal IP-10 expression, in conjunction with elevated IRF-1 expression, was very surprising since LPS-mediated transcription of the IP-10 gene has been correlated with the presence of an ISRE motif in the promoter region (Hamilton *et al.*, 1993; Tebo *et al.*, 1992). One interpretation of this apparent dicotomy is that IRF-1 is not involved in the regulation of the IP-10 gene or the IFN-β gene, whose promoter also contains IRF recognition sequences and is also sub-optimally expressed in tolerized macrophages (Henricson *et al.* 1993; data not shown). Alternatively, IRF-1 may play a role in the transcriptional activation of these genes, but is active only in the presence of additional factors or is somehow inactivated in tolerized cells (Harada *et al.*, 1989; Nelson *et al.*, 1993; Bovolenta *et al.*, 1994).

Combining molecular strategies of gene targeting with the ability to generate transgenic mice has provided invaluable models with which to examine the involvement of a gene of interest in a variety of experimental systems. With this technology, Matsuyama *et al.* (1993) generated mice with targeted disruptions in the DNA binding domains of either IRF-1 or IRF-2 genes. The availability of IRF-1 and IRF-2 "knock-out" mice (IRF-1$^{-/-}$ and IRF-2$^{-/-}$) enabled us to assess the roles of IRF-1 and IRF-2 genes by comparing LPS-

inducible gene expression in macrophages derived from these mice and the control C57BL/6 strain.

Mice with targeted disruption of the IRF-1 gene are reported to have normal numbers and distribution of immune cells except for cells that were TCR$\alpha\beta^+CD4^-CD8^+$ (Matsuyama *et al.*, 1993). This phenotypic characterization included analyses of Mac-1^+ (a cell surface marker present predominantly on macrophages; Springer *et al.*, 1979), suggesting that macrophage populations are largely unaltered in IRF-$1^{-/-}$ mice. Reduced levels of TCR$\alpha\beta^+CD4^-CD8^+$ cells were found in the peripheral blood, spleen, thymus, and lymph nodes (Matsuyama *et al.*, 1993).

Previous studies have implicated IRF-1 in the regulation of poly(I):poly(C)-induced IFN-β, IFN-induced human HLA-B, and iNOS gene expression induced by LPS and IFN-γ (Matsuyama *et al.*, 1993; Girdlestone *et al.*, 1993 ; Kamijo *et al.*, 1994; Martin *et al.*, 1994). Our comparison of LPS-inducible gene expression in IRF-$1^{-/-}$ and C57BL/6 macrophages led to the following observed trends: (1) There was no apparent difference in the expression of TNF-α, IL-1β, TNFR-2, IP-10, IRF-2, or ICSBP genes in medium-treated or 1 - 3 h LPS-treated macrophages derived from both strains. This finding strongly suggests that basal levels of IRF-1 do not affect the expression of these genes in murine peritoneal macrophages, despite the presence of IRF recognition sequences in the promoters of the IP-10 and IRF-2 genes (Tebo *et al.*, 1992; Hamilton *et al.*, 1993; Tanaka *et al.*, 1993). Interestingly, lower basal levels of IFN-β mRNA were detected in IRF-$1^{-/-}$ macrophages in 5 out of 6 experiments (data not shown), which would be predicted by the model of autocrine IFN-β and IRF-1 gene regulation described above. Because the 1 - 3 h LPS-induced expression of all genes examined was largely unaltered in IRF-

$1^{-/-}$ macrophages, it appears that IRF-1 is not involved in the initial steps of LPS signaling and is not a primary transcriptional activator of the immediate-early genes. (2) LPS-inducible TNF-α gene expression was the least affected by IRF-1 gene targeting, and thus, IRF-1 is not an important regulator of LPS-inducible TNF-α. (3) The expression of LPS-inducible IL-1β, IP-10, TNFR-2, ICSBP, IRF-2, and IFN-β genes was consistently lower in IRF-$1^{-/-}$ macrophages compared to the C57BL/6 controls at later time points (i.e., after 4 - 6 h). This finding, in conjunction with the earlier observation that LPS-induced IRF-1 mRNA levels peak within this time frame, suggests that IRF-1 may regulate the expression of these genes. If this is so, it is not clear whether IRF-1 acts directly (i.e., by binding promoter elements) or indirectly (i.e., through the induction of other regulatory gene products), as IRF-1 mRNA accumulates as early as 30 min (data not shown). However, decreased expression of IFN-β mRNA at later time points is consistent with the observation that post-trancriptional regulation accounts for the initial accumulation of LPS-induced IFN-β mRNA (Gessani *et al.*, 1991). Previous studies have shown that LPS-inducible IL-1β mRNA is regulated by NF-κB sites (Hiscott *et al.*, 1993) and LPS-inducible IP-10 mRNA is regulated by both NF-κB and ISRE sites (Hamilton *et al.*, 1993). Recently, IRF-1 was shown to associate with the p50 subunit of a p50/p65 NF-kB complex to regulate cytokine-induced VCAM 1 (vascular cell adhesion molecule 1) gene expression in endothelial cells (Neish *et al.*, 1995). The promoter region of the VCAM 1 gene is similar to the IP-10 promoter in that both NF-κB and ISRE elements are present, so by extension, it is possible that following an initial LPS-stimulated, IRF-1-independent induction of IP-10 mRNA, continued expression is mediated by an IRF-1/NF-κB complex (note that LPS-inducible cytokines such as TNF and IL-1 also induce NF-κB; Narumi *et al.*, 1992; Schütze *et al.*, 1994; Johns *et al.*,

1994). Of this sub-panel of genes, the LPS-induced expression of only IRF-2 and ICSBP genes requires protein synthesis. Promoter analysis of the murine ICSBP gene reveals no conventional IRF recognition sequence (Kanno *et al.*, 1993), and therefore, it seems unlikely that IRF-1 directly activates LPS-induced ICSBP transcription. In contrast, the presence of IRF recognition sequences in the IRF-2 promoter (Tanaka *et al.* 1993), suggests the possibility that IRF-1 may act directly to regulate the IRF-2 gene. Supporting this possibility is the finding of decreased IRF-2 binding to DNA detected in IRF-$1^{-/-}$ fibroblasts (Matsuyama *et al.*, 1993).

We next compared LPS-inducible gene expression in endotoxin-tolerized IRF-$1^{-/-}$ and control C57BL/6 macrophages. The data shown in Table II indicate that in macrophages pre-treated with LPS to induce a state of endotoxin tolerance, the expression of IRF-2, and IFN-β genes were the most dependent on IRF-1. In contrast, almost 6-fold higher levels of IL-1β mRNA (near the maximum level exhibited by in LPS-stimulated, non-tolerized IRF-$1^{-/-}$ macrophages) were detected in endotoxin-tolerized IRF-1-/- macrophages than in endotoxin-tolerized C57BL/6 controls (i.e., prior to "challenge" with LPS). Thus, it appears that in addition to promoting the expression of IL-1β at earlier times, IRF-1 may also be involved in the mechanism that eventually shuts down IL-1β gene expression in endotoxin-tolerized macrophages. Down-regulation of the TNF-α gene occurs by a different (IRF-1-independent) mechanism (since comparable levels are detected in tolerized IRF-$1^{-/-}$ and C57BL/6 macrophages), which is consistent with the earlier observation that TNF-α gene expression was the least affected by targeted disruption of the IRF-1 gene. Whereas the LPS-induced regulation of both TNF-α and IL-1β genes has been shown to involve NF-κB sites (Drouet *et al.*, 1991; Shakhov *et*

al., 1990; Collart *et al.*, 1990; Hiscott *et al.*, 1993), our studies suggest that only IL-1 β gene expression may involve IRF-1. Further complicating an understanding of LPS-induced regulation of TNF-α and IL-1β is the recent finding that macrophages derived from mice with targeted disruptions of the p50 gene express normal levels of TNF-α and IL-1β in response to LPS (Sha *et al.*, 1995), thus implicating alternative pathways, perhaps involving AP-1 as suggested earlier (Bensi *et al.*, 1990; Rhoades *et al.*, 1992). The data shown in Figure 12, indicates that no gene was differentially or appreciably re-induced by LPS, which suggests that IRF-1 is not required for either the induction or maintenance of endotoxin tolerance. To date, the molecular mechanisms that underlie endotoxin tolerance are poorly understood. Early LPS signaling events such as the tyrosine phosphorylation (TP) of MAP kinases, which will be discussed below, do not occur in endotoxin-tolerized macrophages re-stimulated with LPS (Dong *et al.*, 1993). However, LPS-induced nuclear mobilization of NF-κB has been shown to occur independently of TP (Delude *et al.*, 1994), and a recent study has demonstrated that LPS stimulation of endotoxin-tolerized macrophages results in the predominant mobilization of NF-κB p50/p50 homodimers to the nucleus (in contrast to the predominance of p50/p65 heterodimers mobilized in non-tolerized macrophages). Perhaps this accounts for the inability of endotoxin-tolerized macrophages to re-induce NF-κB-dependent genes upon secondary challenge with LPS (Ziegler-Heitbrock *et al.*, 1994).

Flow cytometric analyses of the mice generated with targeted disruption of the IRF-2 gene revealed no apparent abnormalities in thymus, lymph node, and spleen cell populations (Matsuyama *et al.*, 1993). In contrast, bone marrow cell populations were abnormal. Bone marrow derived Thy-1$^+$

and $B220^{high}$/surface IgM^{+} cells (mature T cells and B cells, respectively) were decreased in number, 20-50-% and 30-70%, respectively. In addition, peripheral blood analyses revealed low levels of circulating IgG2a. Colony formation by bone marrow derived B cells in response to LPS was drastically reduced to 30% of the C57BL/6 controls. Colony formation of bone marrow-derived cells in response to IL-3, IL-7, and CSF-1 (colony stimulating factor-1) was reduced to 40% of the C57BL/6 controls. Thus, IRF-$2^{-/-}$ mice have altered hematopoiesis that is expressed in multipotent progenitors, pre-B cells, and macrophages. In this regard, since no analysis of GM-CSF promoted colony formation was made, it is possible that the populations of IRF-$2^{-/-}$ macrophages may be skewed, with respect to the ratio of GM-CSF- to CSF-1-derived macrophages. Of potential relevance, then, is the previous finding that bone marrow-derived colonies formed in the presence of GM-CSF or CSF-1 produce preferentially IL-1β or IL-1α, respectively, in response to LPS, (Witsell and Schook, 1991).

Previous studies have implicated IRF-2 in the regulation of the murine IFN-β gene, the human IL-4 gene, and the murine iNOS gene (Harada *et al.*, 1989; Li-Weber *et al.*, 1994; Salkowski *et al.*, 1995). Our comparison of LPS-inducible gene expression in IRF-$2^{-/-}$ and C57BL/6 macrophages led to the following observed trends: (1) There was no difference in basal level expression of any gene examined, with the possible exception of the IFN-β gene which was expressed at slightly higher levels in IRF-$2^{-/-}$ macrophages in four separate comparisons (data not shown). In contrast, IRF-$2^{-/-}$ fibroblasts were found to contain no detectable basal level expression of IFN-β mRNA; however, these results were derived from Northern blot analyses (Matsuyama *et al.*, 1993). The expression of TNFR-2 mRNA was lower in the IRF-$2^{-/-}$ macrophages essentially throughout the time course, suggesting that

basal levels of IRF-2 may regulate the expression of a gene product(s) that is involved in TNFR-2 mRNA induction by LPS. [Note that IRF-2 mRNA is not induced by LPS until 4 -6 h; Figure 2] In consideration of all genes examined, our data suggests that basal levels of IRF-2 are not important negative regulators of LPS-inducible genes in unstimulated macrophages. (2) IRF-1 and IFN-β (data not shown) gene expression was very similar in LPS-stimulated macrophages derived from IRF-$2^{-/-}$ and C57BL/6 control mice, and thus, IRF-2 is not critical in the regulation of these genes over the 8 h time course. (3) TNF-α, IL-1β, IP-10, and ICSBP gene expression was lower after 4 h of LPS stimulation in IRF-$2^{-/-}$ macrophages, compared to the C57BL/6 controls. The requirement of 4 h of LPS stimulation to detect differences in gene expression is consistent with the delayed onset of LPS-inducible IRF-2 gene expression (Figure 2). It is intriguing that loss of a putative negative regulator of transcription (IRF-2) would result in reduced levels of gene expression. Perhaps protein-function analyses *in vitro* create artificial environments, such that the findings do not always extend to whole cell systems. Alternatively, perhaps loss of one negative regulator (IRF-2) results in an overexpression of yet another "back-up" negative regulator that recognizes the same DNA sequences, such as a yet unidentified or uncharacterized new IRF family. In support of the existence of other IRF family members is the recent discovery of Pip, a novel IRF family member that exhibits extensive sequence homolgy to repressor ICSBP (Eisenbeis *et al.*, 1995).

We next compared LPS-inducible gene expression in endotoxin-tolerized IRF-$2^{-/-}$ and control C57BL/6 macrophages. The data shown in Table III indicate that the expression of IP-10, ICSBP, TNFR-2, and IRF-1 genes

were the most dependent on IRF-2 during endotoxin tolerance. Again, the mechanism by which the loss of a putative regulator (i.e., IRF-2) results in lower levels of gene expression, is presently unclear. TNF-α gene expression was mostly refractory to IRF-2 function, while a two-fold increase in IL-1β was observed. The most striking difference in the gene expression patterns of endotoxin-tolerized IRF-$2^{-/-}$ and C57BL/6 control macrophages, was the finding that IFN-β levels in endotoxin-tolerized IRF-$2^{-/-}$ macrophages were roughly seven-fold higher than the C57BL/6 controls. This finding is consistent with previous studies that demonstrated that IRF-2 is a negative regulator of IRF-1-mediated IFN-β gene expression (Table II; Harada *et al.*, 1989).

In summary, our studies with the IRF-$1^{-/-}$ and IRF-$2^{-/-}$ macrophages have confirmed and extended our hypothesis that IRF-1 and IRF-2 are involved in the regulation of LPS-inducible genes. Our data also suggest that, despite their involvement in LPS-iducible gene regulation, neither IRF-1 nor IRF-2 appear to mediate immediate-early LPS-inducible gene expression or the induction of endotoxin tolerance. Interestingly, targeted disruption of either the IRF-1 or IRF-2 gene resulted in LPS-inducible gene expression patterns that were surprisingly similar. For example, IRF-$1^{-/-}$ and IRF-$2^{-/-}$ macrophages expressed lower levels of IL-1β, IP-10, TNFR-2, and ICSBP mRNA in response to LPS. In addition, neither transcription factor was a major regulator of LPS-inducible TNF-α gene expression. Furthermore, endotoxin-tolerized IRF-$1^{-/-}$ and IRF-$2^{-/-}$ macrophages expressed consistently lower levels of IP-10, ICSBP, and TNFR-2 mRNA, and again, disruption in neither gene had a substantial effect on TNF-α regulation. In contrast, there were also differences in LPS-inducible gene expression patterns observed

between LPS-stimulated IRF-$1^{-/-}$ and IRF-$2^{-/-}$ macrophages. For example, IRF-1 and IRF-2 were found to differentially regulate IFN-β gene expression (especially, in endotoxin-tolerized macrophages) in a manner consistent with previous observations. The finding that IRF-1, and to a lesser extent, IRF-2, may regulate LPS-inducible IL-1β gene expression was a surprising result because there has been no suggestion that IRF recognition sequences may be involved in the regulation of IL-1β promoter activity. This finding suggests that (1) the IRFs participate indirectly in IL-1β gene regulation (i.e., by regulating the expression of other gene products that regulate directly IL-1β promoter activity) or (2) other factors that may bind to IRF-1, or IRF-2, and alter their target sequence specificities and/or DNA binding affinities are potentially involved. Precedence for such a possibility exists in a recent study (Sharf *et al.*, 1995) that has mapped the regions in the carboxy-terminus of ICSBP that mediate the interactions with IRF-1 and IRF-2 that have previously been shown to enhance IRF-2 binding to DNA and decrease IRF-1 binding to DNA (Bovolenta *et al.*, 1994). In any event, our data indicate that both IRF-1 and IRF-2 contribute to nuclear regulation of LPS-inducible gene expression.

Cytoplasmic Regulation of LPS-inducible Gene Expression: The Effects of Ser/Thr Phosphatase Inhibitors, Calyculin A and Okadaic acid, on LPS-Induced Signaling

Previous studies have demonstrated that LPS signaling in macrophages leads to rapid protein phosphorylation and dephosphorylation on serine, threonine, and tyrosine residues (Weiel *et al.*, 1986; Weinstein *et al.*, 1991). Regulation of phosphorylation events involves a balance between the activities of protein kinases and protein phosphatases (Tan, 1993;

reviewed in Hunter, 1995). In this study, we used calyculin A and okadaic acid (OA), two potent inhibitors of protein phosphatases (PP) 1 and/or PP2A, two of the most abundant ser/thr phosphatases in the cell (Cohen, 1989; Suganuma *et al.*, 1990; Cohen *et al.*, 1990), to investigate the potential role of ser/thr phosphatases in LPS signal transduction.

The experiments described above showed that low levels (relative to LPS-inducible levels) of TNF-α, IL-1β, IFN-β, IRF-1, and IP-10 mRNA were induced in macrophages after treatment with calyculin A alone (Figure 20, 21). Thus, it is plausible that macrophage transcription factors are maintained in inactive states by ser/thr phosphatases. In support of this hypothesis are three potentially relevant findings: (1) Calyculin A has been shown to induce phosphorylation of transcription factor *egr*-1 and NF-κB inhibitory protein IκB-α (described above; Cao *et al.*, 1991; Sun *et al.*, 1995); (2) Calyculin A has been shown to induce PKC translocation (Gopalakrishna *et al.*, 1992), perhaps initiating PKC-dependent transcriptional activation; and (3) An inhibitory peptide of PP1 has been shown to increase phosphorylation and activity of CREB (Alberts *et al.*, 1994).

Figure 20 shows that pre-treatment of macrophages with calyculin A for one hour prior to LPS stimulation (-1 h) resulted in a gene expression pattern no different than that achieved by calyculin A alone. Thus, calyculin A inhibited the LPS-induced expression of each immediate-early gene examined. This finding suggests that functional LPS signal transduction, leading to gene expression in macrophages, is dependent upon ser/thr phosphatase activity. Whether this phosphatase activity is constitutively present in resting macrophages, or is induced or activated upon LPS stimulation, is presently unclear; however, identification of the

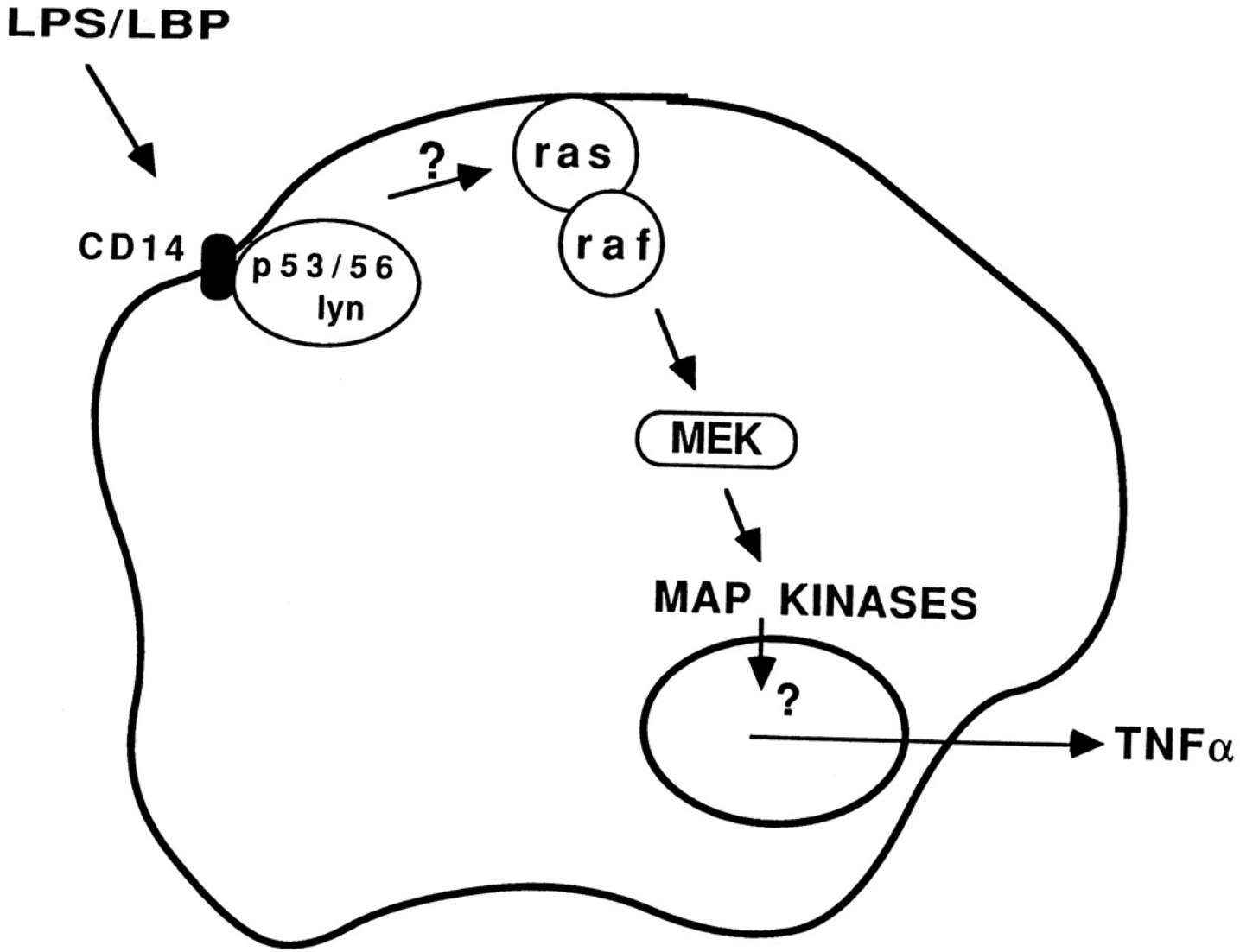
phosphatase(s) involved in this signaling pathway should provide an invaluable target for pharmacologic intervention in septic shock, and help to delineate further the complex intracellular response to LPS. Addition of calyculin A simultaneously with or 1.5 h after LPS stimulation did not inhibit LPS-induced TNF-α, IL-1β, or IFN-β gene expression, but substantially lowered mRNA levels of IP-10, IRF-1, and TNFR-2. One possible interpretation of this finding is that a "late" phosphatase activity regulates an event proximal to transcription, since immediate-early gene transcripts are already induced by 1.5 h (Tannenbaum *et al.*, 1988; Barber *et al.*, 1994; data not shown). TNF and IFN bioassays performed on macrophage culture supernatants, shown in Table VI, supported the calyculin A-mediated inhibition detected at the mRNA level. Macrophages pre-treated with calyculin A 1 h prior to the addition of LPS exhibited bioactivities for TNF and IFN that were suppressed, whereas supernatants from macrophages treated with calyculin A 1.5 h after the addition of LPS, showed TNF and IFN bioactivities that were not measurably different from macrophages treated with LPS alone. Thus, calyculin A-sensitive phosphatases appear not to be required post-transcriptionally. This result is consistent with the recent demonstration that human CSBP1 and CSBP2, and perhaps by extension, murine p38 (due to its high homology with CSBP2), regulate cytokine translation (see discussion below). Taken collectively, the data presented in Figure 20, Tables V, and Table VI suggest that the LPS signaling pathway(s) has (have) at least two ser/thr phosphatase requirements, distinguishable by their time-dependent sensitivities to calyculin A.

Prior to the studies presented herein, it was hypothesized that TP of MAP kinases was required for TNF-α production (described above). A model depicting

this hypothesis is shown in Figure 33, which also depicts the involvement of src-kinases (e.g., *lyn*) and the Ras/Raf-1/MEK/MAP pathway in LPS signaling (described in the Introduction), relative to their suggested temporal positions. Because pre-treatment of macrophages with calyculin A inhibited LPS-induced TNF-α (mRNA and protein), we also analyzed its effect on LPS-induced TP. Figures 22 and 23 show that stimulation of macrophages with calyculin A alone induced TP of three species with the same molecular weights as those seen with LPS, concurrent with the tyrosine dephosphorylation of a ~61 kD protein. To our knowledge, this is the first example of tyr phosphorylation/dephosphorylation regulation by a calyculin A-sensitive phosphatase(s). Furthermore, calyculin A, in the presence or absence of LPS prolonged the tyr phosphorylated states of the three 40 - 45 kDa proteins (Fig. 22).

To confirm serologically that the three co-migrating tyrosine phosphorylated species induced by calyculin A were the same MAP kinases induced by LPS, the identical immunoblot was stripped and reprobed with an antibody that is specific for *erk-1* and *erk-2*. Figure 22 (lower panel) illustrates that at 15 and 30 min after LPS stimulation, *erk-1* and *erk-2* exhibited slower mobilities than observed in medium-treated controls. The slower mobility isoforms have been described previously and have been associated with increased MW, delayed elution from Mono Q anion-exchange columns, and increased kinase activity toward a myelin basic protein substrate, each of these properties considered to be the result of increased protein phosphorylation (Cooper and Hunter 1985; Posada *et al.*, 1991; Weinstein *et al.*, 1992). After 60-90 min of LPS stimulation, a reappearance of the lower MW species, i.e., the dephosphorylated forms, occurred, which coincided exactly with the disappearance of TP. In calyculin A-treated macrophages, *erk-1* and *erk-2* also exhibited slower

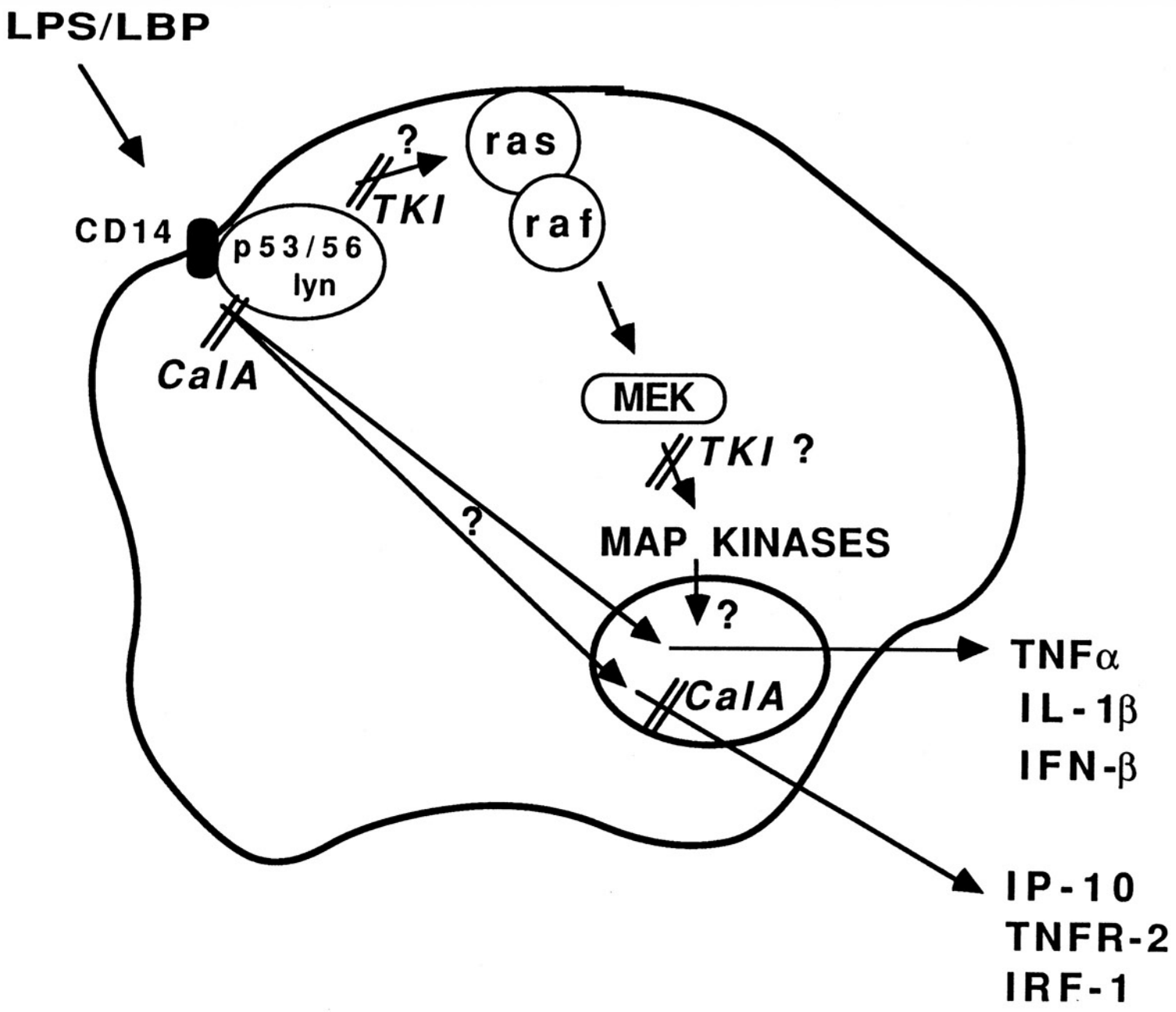
Figure 33. Model of LPS signaling illustrating the proposed participation of lipopolysaccharide-binding protein (LBP), CD14, *lyn*, *ras*, *raf*, MAP kinase kinase (MEK), and MAP kinases in the expression of LPS-inducible TNF-α mRNA.


LPS/LBP
CD14
p53/56
lyn
?
ras
raf
MEK
MAP KINASES
?
TNFα

mobilities than observed in medium-treated controls and were indistinguishable from those present in the 15 min LPS-treated sample. Taken in conjunction with the evidence of TP shown in the upper panel, we conclude that calyculin A induces the TP of MAP kinases *erk-1* and *erk-2*. In macrophages stimulated with both calyculin A and LPS, the higher MW forms of *erk-1* and *erk-2* predominated and the reappearance of the lower MW forms did not occur, in contrast to samples treated with LPS only. This result supports the finding of prolonged TP (Figure 22, upper panel) in the presence of calyculin A and strengthens the notion that calyculin A regulates the same *erk-1* and *erk-2* proteins that have been well described in LPS-treated macrophages. Furthermore, the data suggest that calyculin A either blocks or exerts a dominant negative effect over an LPS-inducible mechanism that normally leads to MAP kinase dephosphorylation. One such mechanism may involve a protein similar to the recently described mitogen-activated, "dual-specificity" MAP kinase phosphatases, MKP-1 or MKP-2 (Sun *et al.*, 1993; Misra-Press *et al.*, 1995).

The model depicted in Figure 33 can thus be modified to allow for integration of our data in the context of previously published findings. Figure 34 illustrates that ser/thr phosphatase activity is required very early in the LPS signaling pathway, upstream of TP of MAP kinases and any branches in the pathway that culminate in immediate-early gene expression. This model is consistent with our observation that pre-treatment with calyculin A (Fig. 20, 21) was required to block the entire LPS signal transduction pathway, as evidenced by the inhibition of every gene examined. That a subset of the panel of immediate-early, LPS-inducible genes is also sensitive to a later addition of calyculin A, implicates a second ser/thr phosphatase requirement for the expression of these genes. Where the branch(es) in the pathway (that dissociate the two subsets of genes) occurs is presently unclear. Furthermore,

Figure 34. Model of LPS-induced signaling modified from Figure 33 to accomodate the data derived from the use of tyrosine kinase inhibitors (TKI) and caylculin A (CAL A). Potential proteins implicated in these pathways include lipopolysaccharide-binding protein (LBP), CD14, *lyn*, *ras*, *raf*, MAP kinase kinase (MEK), and MAP kinases. The subsets of genes induced by the two Cal A-sensitive pathways depicted are TNF-α, IL-1β, and IFN-β and IP-10, TNFR-2, and IRF-1, respectively.


LPS/LBP
CD14
p53/56
lyn
?
TKI
ras
raf
CalA
MEK
TKI ?
MAP KINASES
?
?
CalA
TNFα
IL-1β
IFN-β
IP-10
TNFR-2
IRF-1

the finding that tyrosine phosphorylated MAP kinases clearly predominate in the situation where calyculin A pre-treatment of macrophages inhibits LPS-induced expression of the entire panel of genes (Fig. 20, 21; -1 h), strongly suggests that activation of MAP kinases is not sufficient for the optimal (LPS-comparable) expression of these genes. Thus, cooperation with another intracellular pathway(s) may be required to get the complete nuclear response induced by LPS. Tyrosine kinase inhibitors (TKI) likely effect the *src*-related tyrosine kinases (Levitzki and Gazit, 1995), that, given their temporal position in the LPS signaling pathway, may control any number of downstream events, and therefore, the observations that TKI simultaneously block TP of MAP kinases and TNF production, may reflect two independently regulated events. The strongest piece of evidence, to date, that the MAP kinases are involved in LPS-induced TNF production, is the finding that inhibitors of CSBPs interfere with the translation of LPS-inducible TNF-α and IL-1β (Lee *et al.*, 1994). As described above, dominant negative inhibitors of Ras and Raf-1 were shown to inhibit LPS-inducible TNF-α promoter activity (Geppert *et al.*, 1994). Because Ras and Raf-1 have been shown to have activities distinct from activating the MAP kinase pathway, such as activating PLD or PtdIns 3-kinase (described in the Introduction) in the case of Ras, or activating NF-κB in the case of Raf-1 (Li and Sedivy, 1993), data derived from the use of Ras and Raf-1 dominant negative inhibitors are somewhat subject to interpretation and do not necessarily implicate MAP kinases in TNF production. In addition, since MEK-1 does not phosphorylate p38, there must be other upstream activator(s), such as MKK3 or MKK4 (and by extension, MEKK1 or a related protein) that have yet to be implicated in the pathway. Due to the close temporal relationship between calyculin A- and LPS-induced TP, it cannot be determined whether the TP of MAP kinases in our pre-treatment

regimen is due to the action of calyculin A, LPS, or both. Because there was evidence of TP in this situation which, by the model, blocks LPS signaling leading to gene expression, the TP must result from the action of calyculin A alone (Figure 22). Perhaps dual specificity phosphatases, such as MKP-1 (or MKP-2), are also sensitive to calyculin A, which would provide one mechanism to explain the sustained tyrosine phosphorylated states of the MAP kinases after stimulation with calyculin A, with or without LPS.

The results of our experiments with OA, led us to pursue a different series of investigations than those described above with calyculin A. The major difference between okadaic acid and calyculin A is their relative abilities to inhibit PP1 and PP2A. Previous studies have shown that calyculin A completely inhibits the activities of PP1 and PP2A at concentrations of 1 nM, and, while okadaic acid completely inhibits PP2A at 1 nM, ten to fifteen times higher concentrations are required for the complete inhibition of PP1 (Cohen and Cohen, 1989; Suganuma, *et al.*, 1990). This difference is likely to underlie our observation that okadaic acid was less effective at inhibiting total cellular ser/thr phosphatase activity than calyculin A (Figure 24). Nonetheless, it is tempting to speculate that the different results obtained using calyculin A vs. OA may reflect PP1-like activity not inhibited by okadaic acid treatment. It is presently unclear why OA-mediated inhibition of phosphatase activity required more time than calyculin A-mediated inhibition (Figure 24), as both inhibitors are hydrophobic, and thus, can enter intact cells (Cohen and Cohen, 1989; Suganuma, *et al.*, 1990). Perhaps some yet undescribed properties of the macrophage plasma membrane decrease the kinetics of cell entry by okadaic acid.

Our studies indicated that OA evoked a potent inflammatory response in macrophages, as evidenced by the strong induction of TNF-α, IL-1β, IFN-β, IP-10, and IL-6 genes (Figures 25 and 26). Although OA-induced transcriptional activation of TNF, IL-1, IP-10 and IL-6 genes has been previously observed (Sung *et al.*, 1992; Tebo *et al.*, 1994), the discovery that okadaic acid is a potent stimulant for IFN production is a novel finding. Potential mechanisms by which OA may lead to transcriptional activation are suggested by the following observations: (1) Okadaic acid treatment has been shown to result in the phosphorylation of IκB-α (Sun *et al.*, 1995), and therefore, might be expected to result in nuclear translocation of NF-κB.; (2) Okadaic acid has been shown to promote the expression of transcription factors *egr*-1 and c-*fos* (Schönthal *et al.*, 1991; Cao *et al.*, 1992); (3) Okadaic acid has been shown to induce PKC translocation (Gopalakrishna *et al.*, 1992); and (4) Okadaic acid has been suggested to mimic the early signals induced by TNF and IL-1, both shown to induce IL-6 (Guy *et al.*, 1991). Our studies provide the first evidence that the biological effect of OA is not limited to transcriptional activation, as TNF and IFN bioactivity assays revealed substantial production of these cytokines by okadaic acid-treated macrophages (Table IX). In addition, and in contrast to calyculin A, there was no evidence of okadaic acid-induced TP of MAP kinases at the 1.5 and 8 h time points examined (data not shown). Thus, p38 may not regulate OA-induced TNF-α or IFN-β translation. Interestingly, of the cytokines examined in our study, only IL-10 and IL-12 (p40) genes were not induced by okadaic acid treatment alone (Fig. 26), suggesting that transcriptional activation of these cytokine genes is regulated differentially from the other inflammatory cytokine genes.

Okadaic acid treatment had an inhibitory effect on LPS-induced expression of IRF-1,TNFR-2, and IL-12 (p40) genes, no effect on the expression of LPS-inducible TNF-α or IL-6 genes; but, enhanced the LPS-induced expression of IL-1β, IFN-β, and IL-10 genes (Figures 25 and 26). It is interesting that, although basal levels of IL-10 and IL-12 (p40) were not affected by okadaic acid, LPS-inducible levels were (Figure 26). Moreover, OA greatly enhanced the expression of LPS-inducible IL-10 mRNA, while the expression of IL-12 (p40) mRNA remained essentially the same or was slighly depressed (Table VIII). Taken collectively, these data suggest that LPS induces a ser/thr phosphatase activity, not present in resting macrophages, that differentially regulates the balance of IL-10 to IL-12 (p40) gene expression. This finding supports the possibility that regulators of cytokines that promote T_H1- or T_H2- type immune responses (IL-12 and IL-10, respectively; reviewed in Mossman and Coffman, 1989; Moore *et al.*, 1993; Trinchieri, 1995), exist in macrophages and are inducible by certain extracellular stimuli (e.g., LPS).

Because of the powerful inflammatory response elicited in macrophages by okadaic acid, it was surprising to discover that OA is a potent tumor promoter *in vivo* (Suganuma *et al.*, 1988). As described above, okadaic acid has been shown to increase ser/thr phosphorylation levels (Guy *et al.*, 1991) and transcriptional activity (Schönthal *et al.*, 1991; Cao *et al.*, 1992; Sun *et al.*, 1995), and thus, may contribute directly to the uncontrolled growth of induced tumors. However, OA may also affect the function of immune cells involved in tumor surveillance. One of the properties of activated macrophages is the ability to exhibit tumorcidal activity, which has been correlated with the ability of the macrophage to produce $NO^{\bullet}$ (Lorsbach *et al.*, 1993). Therefore, the effect of okadaic acid on the ability of activated

macrophages to produce NO• was also examined. Macrophage activation typically requires two signals (Ruco and Meltzer, 1978), and a synergistic combination of LPS and IFN-γ has been commonly used for this purpose (reviewed in Nathan, 1992). The data presented in Figure 27 shows that okadaic acid alone did not stimulate NO• production, rather okadaic acid potently inhibited the production of NO• by macrophages activated by LPS and IFN-γ. The conversion of arginine and oxygen to citrulline and NO• by activated macrophages is catalyzed by inducible NO• synthase, the product of the iNOS gene (Nathan, 1992). Although iNOS mRNA is actively transcribed in response to stimulation by LPS and IFN-γ, there have been reports of post-transcriptional regulation as well (reviewed in Xie and Nathan, 1994). Our data (Figure 28) indicated that the effect of OA on NO• production was primarily at the transcriptional level, since iNOS mRNA levels were repressed in macrophages treated with LPS, IFN-γ, and okadaic acid. Further supporting this conclusion is the finding that OA also repressed the expression of IRF-1, a transcriptional activator of the iNOS gene (Matsuyama et al., 1993; Kamijo et al., 1994; Martin et al., 1994). The ability of OA to inhibit NO• production in macrophages may provide one mechanism by which OA-promoted tumors avoid macrophage-mediated tumor cytotoxicity *in vivo*. However, the validity of this hypothesis awaits characterization of tumor-associated macrophages derived from the OA-promoted tumors.

From the above discussion, it is clear that the roles of ser/thr phosphatases in LPS-stimulated macrophages are diverse. Calyculin A-sensitive phosphatases appear to shut down the ability of macrophages to respond to LPS, which, of course, would be desirable in the treatment of septic patients. In addition, calyculin A-sensitive phosphatases dissociate at least

two LPS-inducible pathways, and establish that LPS-inducible gene expression is not solely dependent on MAP kinase activation. Okadaic acid, on the other hand, establishes a role for OA-sensitive phosphatases in the regulation of macrophage inflammatory responses: OA-sensitive phosphatases repress the expression of inflammatory genes in resting macrophages. In fact, our data suggests that a significant number of the primary inflammatory genes in macrophages (i.e., TNF-α, IL-1β, IL-6, IFN-β, and IP-10), are all under the control of this phosphatase activity. In addition, OA-sensitive phosphatases may regulate the expression of T_H1- or T_H2-promoting phenotypes (at least when stimulated by LPS), and the ability of activated macrophages to produce NO$^{\bullet}$. What is most striking about these observations is that diverse biological properties of macrophages appear to be quite tangible, and manipulable, by tapping the correct regulatory ser/thr phosphatases.

Regulation of LPS-inducible Gene Expression at the Plasma Membrane: The Potential Contribution of the Ceramide-Activated Pathway to LPS-Induced Signaling

It has long been recognized that TNF, IL-1, and LPS evoke similar cellular responses. For example, TNF and LPS have been shown to exhibit cross-desensitization *in vivo* (Cavaillon *et al.*, 1993), and TNF and IL-1 have been shown to synergize for the toxicity as well as for the induction of LPS-tolerance *in vivo* (Vogel *et al.*, 1988). In addition, LPS, TNF, and IL-1 each synergize with IFN-γ for the production of NO$^{\bullet}$ (Nathan, 1992). LPS, TNF, and IL-1 stimulation result in the nuclear translocation of NF-κB (Narumi *et al.*, 1992; Schütze *et al.*, 1994; Johns *et al.*, 1994), PC hydolysis to form DAG, and the activation of PKC (Grove *et al.*, 1990; reviewed in Adams *et al.*, 1992; Schütze *et al.*, 1994). LPS and TNF stimulation have been shown to activate

p42 MAP kinase and *raf*-1 (Weinstein *et al.*, 1992; Reimann *et al.*, 1994; Winston and Riches, 1995; Belka *et al.*, 1995; Saleem *et al.*, 1995). LPS, TNF, and IL-1 also elicit expression of a common group of genes including TNF-α, IL-6, IRF-1, and *egr*-1 (Fujita *et al.*, 1989; Zhang *et al.*, 1990; Coleman *et al.*, 1992, Saleem *et al.*, 1995), and all three stimulants have been shown to induce the activation of AP-1 and PLA_2 (reviewed in Wright and Kolesnick, 1995). Finally, okadaic acid has been shown to induce the expression of some of the immediate-early LPS-inducible (Figures 25, 26), as well as TNF- and IL-1-inducible (Guy *et al.*, 1991) genes.

Recently, TNF and IL-1 receptor ligation has been shown to activate sphigomyelinase (SMase), a membrane-associated enzyme that generates the newly recognized second messenger, ceramide, from membrane sphingomyelin (Kolesnick and Golde, 1994; Schütze *et al.*, 1994). Ceramide, in turn, activates a ~97 kDa membrane protein kinase, ceramide-activated protein kinase (CAPK; Liu *et al.*, 1994), and a cytosolic ser/thr phosphatase, ceramide-activated protein phosphatase (CAPP; Dobrowsky and Hunnun, 1993). Other signaling pathways have also been shown to activate SMase (e.g. IFN-γ, CD28, 1α,25-dihydroxyvitamin D3; Okazaki *et al.*, 1990; Kim *et al.*, 1991; Boucher *et al.*, 1995). Exogenous Smase or cell-permeable analogues of ceramide have been shown to induce some of the effects described above for LPS, TNF, and IL-1. For example, ceramide and Smase have been shown to activate MAP kinases (Raines, *et al.*, 1993), induce IL-6 gene expression (Laulederkind *et al.*, 1995), and activate PLA_2 (Weigmann *et al.*, 1994). However, controversy exists in the literature as to the ability of ceramide or Smase to induce the nuclear translocation of NF-κB (Schütze *et al.*, 1992; Kuno *et al.*, 1994; Betts *et al.*, 1994; Johns *et al.*, 1994). In any event, a recent

study illustrated a striking similarity between the structures of the lipid A region of LPS and ceramide, and demonstrated that LPS activated CAPK, in a manner that was both CD14-dependent and enhanced by LBP, but ceramide-independent (Joseph *et al.*, 1994). These authors suggested that LPS stimulates cells by mimicking the second messenger function of ceramide.

To assess the role of the ceramide pathway in LPS-induced signaling, the following questions were posed: (1) Is the ability of ceramide to activate macrophages, like LPS, dependent on the expression of the Lps^n allele?; (2) Does ceramide induce the same panel of LPS-inducible genes in Lps^n macrophages?; (3) Is activation of the ceramide pathway subject to the same regulatory mechanisms as the LPS signaling pathway?; and, (4) Do LPS and ceramide exhibit cross-desensitization (i.e., tolerance) *in vitro*?

The results shown in Figures 29 and 30 indicated, unequivocally, that the ability of macrophages to respond to cell-permeable ceramides, or naturally-derived ceramide (i.e., generated from membrane sphingomyelin by Smase), was dependent on the expression of the Lps^n allele, and thus, a functional product of the Lps^n gene. [It should be noted that SMase generated ceramide in both Lps^n and Lps^d macrophages, as assessed by a DAG kinase assay (Amersham, Inc., Arlington Heights, IL) and thin layer chromatography (TLC; data not shown)]. These data strongly support the hypothesis of Joseph *et al.* (1994), that LPS stimulates cells by mimicking the second messenger function of ceramide.

The second phase of our analysis compared LPS-inducible gene expression resulting from ceramide or LPS stimulation. The data shown in Figures 29 and 31, and Table X, indicate that several doses of three cell-

permeable analogues of ceramide stimulated higher levels of IL-1β and TNFR-2, similar levels of TNF-α, IRF-1, and IRF-2, and lower levels of IP-10 and ICSBP gene expression than those induced by 100 ng/ml LPS. A similar pattern of results were obtained following SMase stimulation for 2 - 6 h, and the lower levels of IP-10 mRNA expression (relative to LPS stimulation) were consistently observed over several SMase concentrations (data not shown). Thus, the results cannot be simply attributed to differences in time- or dose-dependencies. Interestingly, macrophages derived from mice with targeted disruptions in CD14 (i.e., they express neither mCD14 nor sCD14), respond to high concentrations of LPS in serum-free media, to induce high levels of TNF-α and IL-1, and low levels of IP-10 mRNA (Perera *et al.*, submitted). This finding is consistent with the possibility that high concentrations of LPS (shown previously to activate cells CD14-independently) intercalate directly into the membrane, resulting in the activation of membrane-associated CAPK, and thus, a ceramide-like pattern of gene expression. In addition, SMase failed to induce IFN production (Table XI), and again, a wide range of Smase concentrations (0.0625 - 2 U/ml) were examined at times as late as 19 h (data not shown). Collectively, these results suggest that ceramide and LPS induce an overlapping, but distinct pattern of gene expression in macrophages, and thus, activation of the ceramide pathway does not mimic LPS signaling; rather, the ceramide pathway may contribute partial signals to the whole LPS response.

The regulation of LPS and TNF (and perhaps by extension, ceramide) signaling pathways show some similarities. For example, both pathways have been shown to depend on tyrosine kinase activity. As described above, TKI suppressed LPS-induced TP of MAP kinases and TNF-α production in

macrophages (Dong *et al.*, 1993; Novogrodsky *et al.*, 1994). TKI have also been shown to inhibit TNF-induced TP of MAP kinase p42 in human neutrophils (Rafiee *et al.*, 1995) and TNF-induced expression of adhesion molecules in human endothelial cells (Weber *et al.*, 1995). Similarly, LPS and TNF signaling pathways depend on tyrosine phosphatase activity. Tyrosine phosphatase inhibitor, PAO (phenylarsine oxide), has been shown to inhibit LPS-induced *lyn* autophosphorylation in murine macrophages (Henricson *et al.*, 1995), and TNF-induced activation of NF-κB (Singh and Aggarwal, 1995). Because the data presented in the second part of the Results section indicated that the LPS signaling pathway was dependent on ser/thr phosphatase activities, we also evaluated the sensitivity of the ceramide pathway to calyculin A. The results shown in Table XII demonstrated that pre-treatment of macrophages with calyculin A inhibited Smase-induced TNF production. The expression of all other Smase-inducible genes examined were also inhibited by pre-treatment with calyculin A (IL-1β, IRF-1, TNFR-2; data not shown). It should also be noted that the ability of Smase to generate ceramide in the macrophages was not affected by the presence of calyculin A (data not shown). The ability of calyculin A to inhibit Smase-induced gene expression may be due to the ability of calyculin A to inhibit the activity of CAPP, which is a PP2A-like ser/thr phosphatase (Dobrowsky and Hunnun, 1993). In any case, both LPS and ceramide pathways are dependent on ser/thr phosphatase activity.

Previous studies have demonstrated that LPS and TNF induce cross-desensitization *in vivo*; however, pre-treatment of human monocytes with TNF *in vitro*, had no effect on LPS-inducible TNF production (Cavaillon *et al.*, 1993). Recent studies have found that TNF signaling is not solely

dependent on activation of the ceramide pathway (Johns *et al.*, 1994), thus, creating the possibility that perhaps pre-treatment with ceramide alone would provide a cross-desensitization signal for LPS. Therefore, as a final comparison of the ceramide and LPS pathways, we examined the abilities of LPS and ceramide to exhibit cross-desensitization *in vitro*. The data shown in Figure XIII indicate that pre-treatment with LPS rendered macrophages tolerant to both LPS and Smase. In contrast macrophages pre-treated with Smase were neither tolerant to LPS nor Smase. [It should be noted that the ability of Smase to generate ceramide was not affected by the induction of endotoxin-tolerance in macrophages; data not shown] These data suggest that the mechanisms that underlie the phenomenon of endotoxin-tolerance involve a critical molecule(s) required for activation of the ceramide pathway. Conversely, activation of the ceramide pathway does not elicit signals that lead to the induction of a tolerant state.

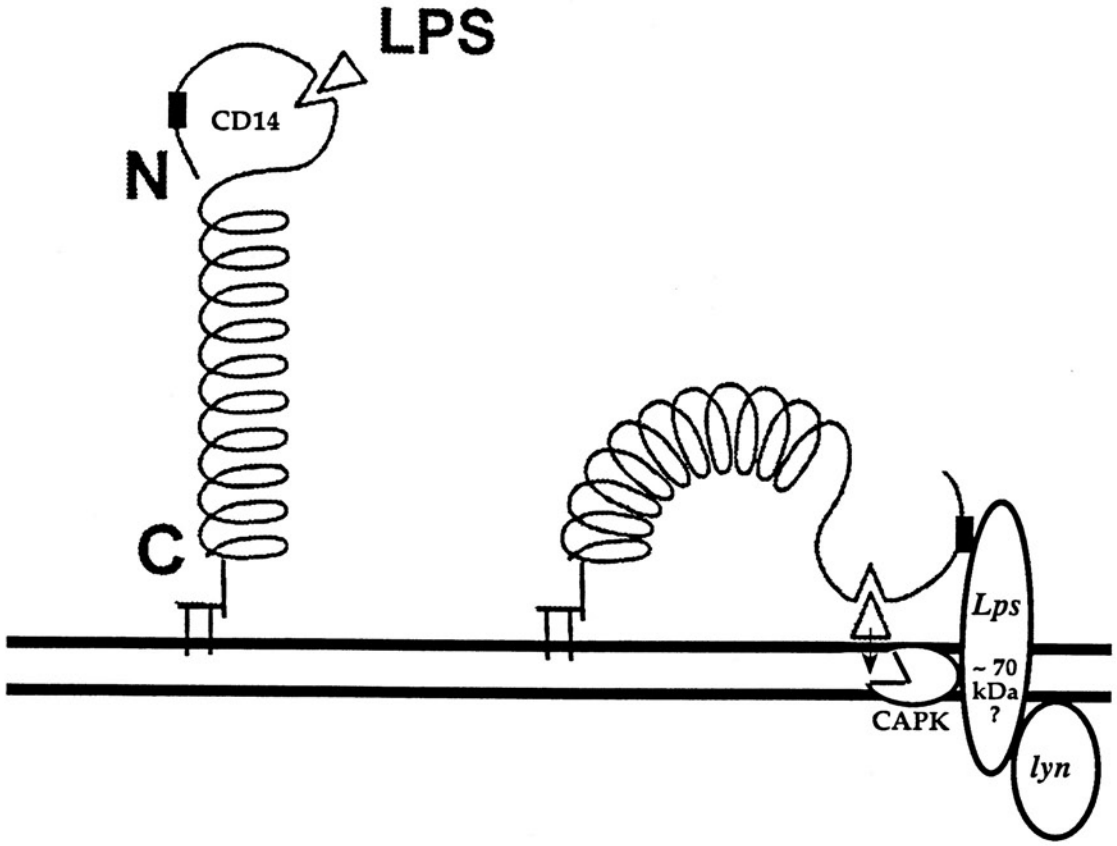
In summary, two findings described above illustrate a strong correlation between ceramide- and LPS-induced signaling: (1) Both signaling pathways are absolutely dependent on the expression of the Lps^n allele; and, (2) The molecular mechanisms required for the induction of endotoxin tolerance utilize critical molecules involved in the ceramide pathway. Considering both observations, it would not be unreasonable at this point to suggest that the common critical molecule is the Lps^n gene product. However these data also reveal two observations that suggest that LPS stimulation of cells cannot result entirely from mimicking the second messenger function of ceramide: (1) Activation of the ceramide pathway does not lead to LPS-comparable expression of IP-10 and ICSBP mRNA or production of IFN; and, (2) Activation of the ceramide pathway does not lead to a state of

desensitization (tolerance) that is characteristic of LPS. Thus, it is more likely that the ceramide pathway contributes a portion of the LPS response.

The latter segment of this research has led to novel findings that enable us to propose a model of LPS signaling at the plasma membrane. This model, shown in Figure 35, is based primarily on the following observations: (1) Membrane expression of CD14 is required for LPS stimulation of 70Z/3 cells,despite the fact that the GPI anchor is dispensible (Lee *et al.*, 1993); (2) Src-related kinase *lyn* co-immunoprecipitates with CD14 in activated human monocytes (Stefanova *et al.*, 1993); (3) LPS-stimulation of C3H/HeJ macrophages does not result in *lyn* autophosphorylation (Henricson *et al.*, 1995); (4) LPS stimulates CAPK in a manner that is both CD14-dependent and enhanced by LBP, but ceramide-independent (Joseph *et al.*, 1994); (5) Activation of the ceramide pathway is dependent on the product of the *Lps* gene (Figures 29 and 30); and, (6) LPS stimulation of macrophages does not solely invoke (mimic) activation of the ceramide pathway (Figure 31, Tables X, XI, XIII).

The model depicts the putative product of the Lps^n gene as a bitopic integral membrane protein (i.e., it contains hydrophilic domains on either side of the membrane; Blobel, 1980). In resting macrophages, the putative Lps^n gene product is associated with CAPK in the membrane, in such as way as to maintain the kinase in an inactive state. The molecular mechanisms predicted by this model, that may mediate CD14-dependent and CD14-independent stimulation by LPS, and activation of the ceramide pathway in macrophages, are described below.

Figure 35. Model of the molecular interactions occurring at the macrophage plasma membrane upon stimulation with LPS. Depicted in the model are proteins CD14, the putative product of the *Lps* gene, ceramide-activated protein kinase (CAPK), and *lyn* kinase. The small black box depicted near the N-terminus of the CD14 molecule represents the amino acids required for signaling, as recently defined by Lee *et al.* (1993).


LPS
CD14
N
C
Lps
~ 70
kDa
?
CAPK
lyn

CD14-dependent signaling. Upon binding of the LBP/LPS complex, CD14 might undergo a conformational change such that an association occurs with the extracellular domain of the putative *Lps*^{n} gene product . This interaction, in turn, induces a conformational change in the *Lps*^{n} gene product that facilitates the interaction of LPS with CAPK, which then results in the dissociation of the *Lps*^{n} gene product (from CAPK) and activation of the kinase. The dissociated *Lps*^{n} gene product mediates activation src-related kinase *lyn* directly or indirectly (via another protein/protein interaction). This model suggests that the *Lps*^{d} allele encodes a mutated protein that does not dissociate from ceramide- or LPS-activated CAPK, and thus, does not mediate *lyn* activation. In addition, this model is consistent with several observations: (1) mCD14 is required for LPS signaling in 70Z/3 cells; (2) src-related kinase *lyn* co-immunoprecipitates with CD14 in LPS-stimulated human monocytes; (3) LPS activates CAPK in a manner that is CD14-dependent and enhanced by LBP; (4) LPS and ceramide signaling is dependent on the *Lps*^{n} gene product; and, (5) LPS signaling stimulates *lyn* autophosphorylation in *Lps*^{n}, but not *Lps*^{d} macrophages.

CD14-independent signaling. High concentrations of LPS stimulate cells in a CD14-independent manner. In this model, high concentrations of LPS may intercalate into the membrane directly to activate the CAPK/*Lps*^{n} gene product complex, essentially by-passing the formation of an LPS/LBP/CD14/*Lps*^{n} gene product complex. Thus, the signals resulting from this stimulatory pathway may lack signals derived from formation of the latter complex. In support of this hypothesis is the recent finding that the pattern of gene expression resulting from stimulation of CD14$^{-/-}$ macrophages in sCD14-free medium containing high concentrations of LPS mirrors that

induced by ceramide (i.e., high TNF-α and IL-1β, but low IP-10 mRNA; Perera *et al.*, submitted).

Activation of the ceramide pathway. In this model, activation of CAPK would be dependent on the functional *Lps*^{n} gene product. This is consistent with our findings that neither ceramide nor Smase stimulated *Lps*^{d} macrophages. It is unlikely that CAPK is the *Lps*^{n} gene product because activation of the ceramide pathway alone, does not completely mimic LPS signaling. It also remains to be seen if CAPK is able to be activated in *Lps*^{d} macrophages. How CAPP may fit into the model is currently unclear; CAPP is activated directly by ceramide, and perhaps also by LPS (due to structural similarity), but due to its cytosolic location, CAPP is unlikely to be a primary target for ceramide and/or LPS in CD14-independent situations.

Why is it, then, that the full array of CD14-dependent LPS signaling events are not elicited in response to ceramide- or CD14-independent LPS-induced stimulation of macrophages? The unequivalent signaling pathways may be explained, in this model, by the lack of formation of the initial LBP/LPS/mCD14/*Lps*^{n} gene product complex. Lee *et al.* (1993) demonstrated that mCD14 was required for LPS signaling of 70Z/3 cells (despite the inability of CD14 to transmit signals itself), implicating the importance of such a complex. It follows, then, that the formation of this complex initiates a "signal(s)" that distinguish(es) ceramide- and LPS- (CD14-independent) induced patterns of gene expression from those elicited in a CD14-dependent LPS-induced fashion. The results of two independent studies suggest that this "signal" may ultimately lead to the translocation of PKC. In contrast to extracellular stimulation with LPS, LPS delivered into the cell via liposomes, induced IL-1 mRNA, but not the translocation of PKC (Bakouche *et al.*, 1992).

Intracellular LPS likely has access to membrane bound CAPK (and cytosolic CAPP), but not mCD14. One conclusion reached by the authors of the study was that extracellular signaling by LPS (i.e., via mCD14) is required for LPS-induced translocation of PKC. In another study, ceramide was shown to inhibit translocation of certain isoforms of PKC (Jones and Murray, 1995). Thus, in both ceramide- and CD14-independent LPS-induced situations, PKC translocation is altered, and therefore, it is possible that the formation of the LBP/LPS/mCD14/*Lps*^{n} gene product complex is required to initiate the signaling events leading to the translocation of PKC. In support of this hypothesis are studies that distinguish, in this model downstream receptor-signaling events (i.e., *lyn* activation) from upstream events (i.e., initial membrane perturbations or "signals" leading to the translocation of PKC). Golenbock and colleagues (Delude *et al.*, 1994) reported that CD14-dependent activation of NF-κB is refractory to TKI. In the model depicted in Figure 35, signals preceding *lyn* activation, such as those initiating PKC translocation and/or releasing CAPK inhibition, would not be expected to be TKI-sensitive, and as described above, PKC has been shown to phosphorylate Iκ-B, the cytosolic inhibitor of NF-κB.

Finally, it is conceivable that the extracellular domain of the *Lps*^{n} gene product, depicted in the model, may be the structural epitope that is recognized by monoclonal antibody 5D3. As such, it would be interesting to see if the agonist activity exhibited by 5D3, mimics the ceramide- or LPS-induced (CD14-dependent) patterns of gene expression.

In summary, the model depicted in Figure 35, has incorporated the results of experiments perfomed in this study with those derived from other studies that have been conducted over that past several years. Thus, the work

presented herein has served to further the understanding of how LPS interacts with the macrophage to elicit the activation of a specific signaling pathway that leads to gene expression and ultimately the production of cytokines. In this regard, these studies may provide the framework for future studies that will eventually identify the *Lps*^{n} gene product, which may, in turn, lead to the development of an effective therapy for use in the treatment of septic shock.

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